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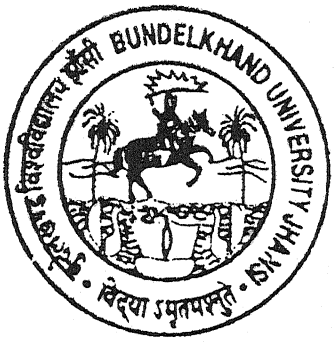
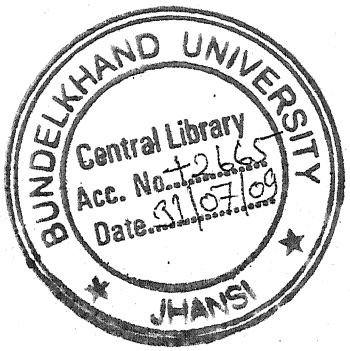
GENOMIC FINGERPRINTING AND DEVELOPMENT OF MOLECULAR PROBES FOR RAPID DETECTION OF *MACROPHOMINA PHASEOLINA*





Thesis Submitted for the Degree of
Doctor of Philosophy
In
Microbiology

By

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Dedicated to my teacher

Prof. Dilip K Arora and my parents

Certificate

This is to certify that the work embodied in the thesis entitled **"GENOMIC FINGERPRINTING AND DEVELOPMENT OF MOLECULAR PROBES FOR RAPID DETECTION OF *MACROPHOMINA PHASEOLINA*"** *is submitted by* **Mr. BANDAMARAVURI KISHORE BABU** *for the award of the degree of* **Doctor of Philosophy (Ph.D.)** *in* **Microbiology** *Bundelkhand University, Jhansi. It is a record of bona-fide research work carried by him under our supervision and guidance. This work has not been submitted elsewhere for a degree / diploma in any form.*

It is further certified that he has worked with us for the period required under the Ph.D. degree ordinance–7 of the Bundelkhand University, Jhansi.

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Declaration

I hereby inform that work presented in this thesis **"GENOMIC FINGERPRINTING AND DEVELOPMENT OF MOLECULAR PROBES FOR RAPID DETECTION OF *MACROPHOMINA PHASEOLINA*"** *entirely my own work and there is no collaboration. It is further certified that as per the best of my knowledge this thesis does not contain any work for which any other university has awarded a degree and diploma.*

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(BANDAMARAVURI KISHORE BABU)

CONTENTS

AFLP	Amplified fragment length polymorphism
APDA	Acidified potato dextrose agar
Approx.	Approximately
BCIP	5-bromo-4chloro-3-indolyl phosphate
bp	Base pair
Cm	Centimeter
Cm^3	Cubic centimeter
DNA	Deoxyribo nucleic acid
dNTPs	Deoxynucleotides
ELISA	Enzyme linked immunosorbent assays
ERIC	Enterobacterial repetitive intergenic consensus
Et.Br	Ethidium bromide
g	Gram
h	Hours
IGS	Inter Genic Spacer region
ITS	Intergenic spacers
Kb	Kilo base
L	Liter
M	Mole
mg ml^{-1}	Milligram per milliliter
Min	Minutes
mL	Millilitre
mm	Millimeter
mM	Millimole
n	Nanometer

°C	Degree celsius
P	Primer
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pH	Hydrogen ion concentration
pmol	Picomol
RAPD	Random amplified polymorphicdna
rDNA	Ribosomal DNA
REP	Repetitive extragenic palindromic
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SDW	Sterilized distilled water
Sec	Seconds
SSCP	Single strand confirmation polymorphism
TAE	Tris-Acetate-EDTA buffer
UPGMA	Unweighted pair group arthimetic average
UV	Ultra violet
V	Volt
V cm^{-1}	Volt per centimeter
W/V	Weight by volume
µg	Micro gram
µL	Microlitre

CHAPTER I

INTRODUCTION

I. **Common diseases caused by *M. phaseolina***

II. **Major cultivated hosts include**

III. **Classification**

IV. **Nomenclature**

V. **Importance of proposed research and objectives**

VI. **Major objectives**

Macrophomina phaseolina (hereafter referred as *M. phaseolina*) is a primarily soil-borne pathogen with wide distribution, varied host range, greater longevity and higher competitive saprophytic ability (Young *et al.* 1982; Sobti and Bansal 1988; Chattanaver *et al.* 1988; Das 1988; Singh *et al.* 1988; Abbaiah and Satayanarayana 1990; Das and Sankar 1990; Osunlaja 1990; Singh *et al.* 1990; Srivastava and Singh 1990; Kaur and Mukhopadhaya 1992; Siddiqui and Mehmood 1992; Mukherjee 1993). It causes disease in more than 400 economically important plants (Mihail *et al.* 1988). The fungus is also associated with seeds and it is shown that infection led to both pre- and post-emergence mortality, causing seed to seedling transmission of the pathogen (Pun *et al.* 1998). The pathogen occurs both inter and intra-cellular. The amount of internal inoculums is directly related with the degree of symptoms expressed (Sharma and Singh 2000).

The pathogen causes wide range of diseases in the arid and semi-arid regions of the world (Reichert and Hellinger 1947; Young 1949; Dhingra and Sinclair 1978). *M. phaseolina* persists in soil as sclerotia formed in infected host tissue and later released in the soil during decaying process (Smith 1969). As a root inhabitant the fungus is widespread in warmer area, invades immature, damaged or senescent tissues; plants are generally attacked at seedling and flowering, when conditions are hot and dry. Infection develops from sclerotia, which can survive for a few years in roots. *M. phaseolina* is widely distributed among the areas with variable soil types and annual rainfall, indicating that this fungus can persist under highly diverse environmental conditions. Many sclerotia were found in decaying plant parts and in the seed coat (Ahmed *et al.* 2001). Primary infection takes place from roots or seeds, starting from seedling stage till harvest. As regard to seed-borne nature, the pathogen is carried

internally or externally; the propagules may be either mycelial fragments or sclerotia. In case of soil-borne phase, the pathogen remains either on the dead organic debris or on the root stubbles, which are left over after the crop harvest. When disease free seeds are sown in such fields, the germinated sclerotia spread mycelia all around. The young hyphae either attack the tender roots or the collar region. Under favourable climate, the infection proceeds further resulting into either root rot or collar rot. High soil temperature (40°C), low soil pH (5.4-6), low soil moisture (8-16%), high humidity (90%) favour such infection and disease development. Stagnation of water, especially where land is low and prevalence of sandy-loam soil is the most favourable condition for faster development of the disease (Wokocha 2000). Considerable research has been carried out on the effect of abiotic factors such as pH, temperature, moisture, C/N ratio, CO_2 concentration, *etc.* on the growth and survival of *M. phaseolina* in soil (Dhingra and Sinclair 1975; Banerjee *et al.* 1982; Gangopadhaya *et al.* 1982; Singh *et al.* 1988; Taya *et al.* 1988, 1990; Bhatti and Kraft 1992). Long periods of drought and hot temperatures are interspersed with rain showers create ideal conditions for fungal pathogenesis (Pederson *et al.* 2000). The study showed that high soil moisture levels were unfavourable for the growth and pathogenicity of *M. phaseolina*, while low soil moisture levels favoured the growth and pathogenicity of the fungus (Sarkar and Pradhan 1999).

M. phaseolina has a limited saprophytic ability because of the antagonism of other soil-borne microorganisms (Norton 1953, 1954; Bhattacharya *et al.* 1985). The diversity of reported hosts and widespread geographic range of fungus suggest that *M. phaseolina* may be quite heterogeneous. Variability among *Macrophomina* isolates has been evident even in the restricted focus of multiple isolates obtained from a single host plant of soybean (Dhingra and Sinclair 1973a) and Urd bean (Jain *et al.* 1973).

The pathogen attacks the root system of the plants and causes dissolution of all tissues except the xylem and symptoms follows wilting by withering and death of aerial parts are characteristics of the disease (Edmunds 1964). The infected roots have abundant mycelia and sclerotia but rarely pycnidia are produced on infected roots (Knox-Davies 1967). Both pycnidiospores and sclerotia have been implicated in the propagation of this fungus. The pathogen is plurivorus, causing ashy stem, blight or charcoal rot; root and stem show destruction of the cortex. The fungus is also the causal agent of the charcoal rot disease of many crops (Mihail 1992). In some cases, as in histological sections of roots infected with *M. phaseolina*, showed destroyed cortical parenchyma, both giant cell and also cortical parenchyma were invaded by mycelia. Inhibition of penetration through the outer cell wall of the upper epidermis may be attributable to an osmiophilic layer below the cell wall. Disruption of the host cell walls and subsequent host cell death was preceded by massive colonization of the host (Joye and Paul 1992).

I. Common diseases caused by *M. phaseolina*

- **Charcoal Rot:** Charcoal rot, induced by *M. phaseolina*, attacks crop at different stages of plant growth to varying degrees. Pycnidia of the fungus are formed, phloem and xylem tissues become brown and the sclerotia of the fungus are found in the soil. The fungus colonizes roots and stalks causing disintegration of tissues while spreading rapidly. It was observed that unfavourable soil moisture, low (40%) as well as high (100%) for sowing crop caused maximum pre-emergence rot, post-emergence mortality was maximum only under conditions of moisture stress (40% soil moisture). The optimum temperature (30-35°C) for growth of the pathogen also favoured maximum disease development under artificial inoculation. The younger the pathogenic culture the more aggressive it was in infecting cowpea seedlings, while the

susceptibility of crop increased with increasing plant age (Payak and Sharma 1978; Seetharama *et al.* 1987; Chandra *et al.* 1995; Sandhu and Singh 1999).

- **Seedling Blight:** It occurs at seedling stage of the crop. Due to infection and rotting of tissues, leaves turn yellowish brown to black. The rot travels through the petiole to the young stem causing blight. Sometimes, necrotic wound develops at the node and cause breaking of young stem at nodal regions (Noble and Richardson 1968).
- **Collar Rot:** It occurs during full growth stage at the collar region of the plant (Jhooty and Brains 1972).
- **Stem Rot:** It occurs on stem at later stages of the crop growth. At elongation stage; Brown to black colored lesions first appears on the margins, which gradually spread. At flowering stage, lesions are formed on inflorescence, capsules become discolored, seeds turn black and small, and sometimes sclerotia appear in the capsules (Noble and Richardson 1968).
- **Damping-off:** It occurs at the emerging stage of the crop. Minute black pinhead spots are the first visible symptoms, which coalesce by spreading up and down over the tender stem, spots later become water-soaked and finally turn brownish to black and sometimes, dark thin streaks also develop on the cotyledons and collar region (Chandra *et al.* 1995).
- **Root Rot:** The taproot of plant is infected directly by the soil inhabitant phase of the pathogen. The plants attack in early stages is recognized by drooping and wilting. The green colour of the stem gradually turns brown to dark brown. The taproot including its branches becomes rusty brown. It has been observed that plants may survive, if only the lateral branches of the roots are infected but in case, taproot is attacked, survival is generally nil. The occurrence of this disease starts from first week of July and continues till harvest (Vasudeva 1960; Noble and Richardson 1968; Jhooty and Brains 1972).

Besides above, *M. phaseolina* causes disease on a large number of plants including vegetables, ornamentals, shrubs and trees (Gangopadhayay *et al.* 1973).

II. Major cultivated hosts include:

Arachis hypogea (peanut)	*Brassica oleracea* (Cabbage)	*Phaseolus* spp.
Capsicum annum (pepper)	*Cicer arietinum* (chick pea)	*Citrus* spp.
Glycine max (soybean)	*Ipomoea batatas* (sweet potato)	*Beta vulgaris*
Solanum tuberosum (potato)	*Vigna unguiculata* (bean)	*Zea mays* (corn)
Sorghum bicolor (sorghum)	*Sesamum indicum* (sesame)	*Fargaria* spp.
Corchorus spp.	*Medicago sativa* (alfalfa)	*Cucumis* spp.
Gossypium spp.	*Helianthus annus* (sunflower)	*Pinus* spp.
Prunus spp.		

The disease is predominantly destructive on cultivated crop plants like cereals and oil seeds. The fungus also infects a wide variety of plants including pulses, oil seeds, sorghum, maize, jute, and vegetables and fruit plants. It has also been reported on onions (Garg and Chauhan 1981), garlic bulbs (Chandra and Tandon 1965), and causes charcoal rot on corn, sorghum, soybean and other economical crop plants (Watanabe *et al.* 1970; Meyer *et al.* 1973). It is one of the main causes of huge losses every year on soybean in India and United States (Sinclair and Gray 1972). In legumes it causes root rot of pigeon pea, chickpea, black gram and groundnut (Samiappan and Vidhyasekharan 1981) and black rot in sweet potato (Garg and Chauhan 1981).

III. Classification

Macrophomina phaseolina belongs to sub-division Duteromycotina, Class Coelomycetes (Mihail 1992).

Order – Sphaeropsidales

Family – Sphaerioidacease

Genus – *Macrophomina*

The genus "*Macrophomina*" contains only one species "*phaseolina*" (Sutton 1980).

IV. Nomenclature

The successive changes in nomenclature created confusion to adapt the correct name of *Macrophomina*. The monotypic genus *Macrophomina* was established by Petrak (1923) as *M. philippinensis*. Subsequently (Ashby 1927) examined the type material of this fungus and compared with several other genera, and established earlier binomial for the fungus as *Macrophomina phaseoli* Maubl. Consequently Ashby proposed the combination *Macrophomina phaseoli* (Maubl.) (Ashby) for *Macrophoma phaseoli* Maubl. and relegated the synonym *M. cajani* P. Syd. and Butler, *M. chorchori* Sawada, *M. sesami* Sewada, *Macrophomina philippinensis* Petrak, *Sclerotium bataticola* Taub., *Rhizoctonia bataticola* (Taub.) Butler and *Dothiorella cajani* (P. Syd. and Butler), Petrak and H. Syd. Goidanich (1947) reviewed the taxonomy of *Macrophomina*. Petrak (1923) named this as *M. phaseolina* Tassi. in the place of *M. phaseoli* Maubl. From 1947 onwards the two names *i.e., M. phaseoli* (Maubl.) (Ashby) and *M. phaseolina* (Tassi.) Goid became well established in psychopathological literature as the cause of charcoal rot of several important crop plants. After 1977, several other names were suggested for the fungus and ultimately *Macrophomina phaseolina* (Tassi.) Goid. was accepted as correct name (CMI description of pathogenic fungi and bacteria no. 275). The sclerotial phase of *M. phaseolina* is known as *Rhizoctonia bataticola* (Thakurji 1979; Punithalingam 1982).

The identification of *M. phaseolina* is usually based on morphological criteria; however, due to wide variations in the phenotype of the isolates these criteria are often not reliable. Pathogenic variability among the isolates or sensitivity to chlorate was utilized to categorize the isolates (Cloud and Rupe 1991; Pearson *et al.* 1987a). Molecular methods have been recently described to resolve genetic variation among the isolates of *M. phaseolina* (Purkayastha *et al.* 2006; Das *et al.* 2006; Jana *et al.* 2005a and Jana *et al.* 2005b). Moreover, these techniques are laborious, time consuming and useful only for grouping of isolates rather than identification. Therefore, a rapid diagnostic technique that can identify and detect *M. phaseolina,* both *in vitro* and *in vivo* conditions is required. Oligonucleotide specific primers or probes targeting the Internal transcribed spacer (ITS) region have been reported to selectively detect several agriculturally important fungi like *Trichoderma, Hypocrea* (Irina *et al.* 2005), *Fusarium* (Edel *et al.* 2000), *Verticillium* (Nazar *et al.* 1991) and *Phytophthora* (Lee *et al.* 1993). However, no markers are available for specific detection of *M. phaseolina.* Screening of the GenBank for ITS sequences of *M. phaseolina* revealed the existence of very few sequences that showed some degree of variation among the isolates. Sequence variation in the rRNA genes may allow the use of these genes as targets for differential amplification. This prompted us to carry out amplification of ITS region, followed by Restriction fragment length polymorphism (RFLP), sequencing and analysis of the ITS region for identifying the conserved and variable motifs.

This study was therefore aimed at developing specific primers and oligonucleotide probe (within the ITS region) and subsequent evaluation of their efficiency for identification/detection of *M. phaseolina* under *in vitro* conditions. Attempts to study the genetic diversity in relation to geographical distribution and pathogenic variation will facilitate to develop effective/resistance cultivars. Little is known about the genetic complexity of *M. phaseolina* population in India

or about the origin and spread of the pathogen *i.e.*, whether, it arose as a single lineage in one location or arose independently in several locations. Various recent studies were developed to the genetic and pathogenic variability of *M. phaseolina*. Significant advances in molecular detection and differentiation of *M. phaseolina* has been achieved using RFLP, RAPD and AFLP analysis (Mayek-perez *et al.* 2001; Su *et al.* 2001; Purakayastha *et al.* 2006; Reyes- Franco *et al.* 2006). The lack of strong correlation between genotype and geographical origin suggests a high diversity level within and among the population of *M. phaseolina* (Jana *et al.* 2005a, b). So far none of these methods have however been able to differentiate isolates of *M. phaseolina* from specific hosts or geographic locations. RAPD analysis has many advantages as a means of characterizing genetic variability such as speed, low cost and minimal requirement for DNA. Major polymorphisms in RAPD pattern indicate genetic distinctness and can be used to distinguish unrelated groups.

The current study was undertaken to elucidate the genetic diversity of *M. phaseolina* isolates of Indian origin by using RAPD molecular markers to provide insight into its genetic relation between molecular diversity and geographic origin. In this study we also described the use of single short repetitive primer in rep-PCR DNA fingerprinting technique to evaluate the genetic diversity among *M. phaseolina* isolates.

V. Importance of proposed research and objectives

Although references are scanty, the impression exists that charcoal rot caused by *M. phaseolina* has progressed in fast in the semi-arid zone of the India. Pervious studies on *M. phaseolina* have investigated variation in morphology and pathogencity among isolates of various hosts. The genus *Macrophomina* consists of only one species *phaseolina* comprising thousands of strains/isolates/biotypes/races/pathotypes. Considering the morphological and

cultural characters, virtually there is no difference between thousands of pathogenic and non-pathogenic isolates/pathotypes of *M. phaseolina.* This pathogenic fungus was selected in this study because of its broad host range, and its ability to develop acquired tolerance against pesticides and hybrid host varieties. Another reason for the selection of this pathogen was that it causes diseases to about 500 plants among which charcoal-dry root rot of chickpea (*Cicer arietinum*), is one of the most common diseases in South East Asian countries and severely limits chickpea production. A reliable, sensitive and rapid method for the detection, characterization, identification and monitoring of this fungus *in vitro*, in greenhouse and in field conditions is needed. Therefore, the overall objectives of this research was to compare different morphological and molecular techniques for the characterization and identification of *M. phaseolina* and to develop authentic, speedy and simple assays for characterization, identification, detection, screening and monitoring of population of pathogenic as well as non-pathogenic *M. phaseolina.* Surprisingly, no comprehensive scientific investigation was carried out to compare the utility of morphological and molecular techniques in characterization and identification of different isolates of *M. phaseolina* present in soil/seed lots or which infects different plants. In plant pathology early identification of the causative agent of disease is paramount in order to recognize the pathogen, and implement regulations involving control and quarantine. Morphological, ecological and pathological traits of a species is usually defined from pertinent characters, most of which will relate directly to functional and structural attributes. The development of morphological and molecular tools to characterize and detect this fungus in infected plant parts, rhizosphere or from field soils is main goal of this study.

VI. Major objectives:

Objective 1

To investigate different morphological and cultural techniques for the isolation, characterization and identification of *M. phaseolina* from a various host range and different agro-climatic regions of India.

Objective 2

To develop species-specific primers and molecular probes for diagnosis of *M. phaseolina* and evaluation of ITS (ITS1/ITS4) sequences to develop PCR-based assays for the characterization and study of genetic variation of different isolates of *M. phaseolina.*

Objective 3

To characterize the heterogeneity in different isolates of *M. phaseolina* using Randomly amplified polymorphic DNA (RAPD–PCR) gene sequences.

CHAPTER II

REVIEW OF LITERATURE

I. **Morphological and Cultural Characterization**

II. **Molecular methods for the characterization and identification of Fungi**

1. PCR-Based Methods
2. Molecular targets used for the detection of Fungi
3. Ribosomal DNA Gene Cluster
4. 18S rRNA Gene as Target
5. 5. 28S rRNA Gene as Target
6. Intergenic Transcribed Spacer
7. Random Amplified Polymorphic DNA
8. Similarity coefficients

REVIEW OF LITERATURE

Macrophomina phaseolina (Tassi) Goid. is a major soilborne fungal pathogen that infects many agronomic, horticultural, and ornamental crops (Mihail *et al.* 1992). It is unusual, that a wide variety of both monocots and dicots are parasitized. Diseases caused by *M. phaseolina* are sometimes referred to as 'charcoal rot' on account of small, black, macroscopically visible sclerotia that form in shredded, parasitized host tissue and cause an appearance of charcoal. Primary infection by *M. phaseolina* usually occurs in roots, but pathogenesis and sclerotium formation may extend above ground. Collapse and death of infected plants usually is favored by hot and dry conditions, but numerous sclerotia also may form in live host tissues without visible disease symptoms. Sclerotia are the resting structures of this pathogen. The most interesting thing about this fungus is that it has only one species (Sutton 1980), but thousands of isolates. The biggest problem before mycologists/plant pathologists is to identify/detect thousands of different isolates of this fungus from the cultures, in infected roots, soil and seed lots. Identification, diagnosis and detection of *M. phaseolina* is very difficult because isolates are morphologically very similar. Various scientists have adapted different methods to distinguish *M. phaseolina* isolates. However, two prominent methods which are applied in this piece of research for detection are: (i) morphological and cultural characterization (ii) PCR-based molecular methods for the characterization and identification.

I. Morphological and Cultural Characterization

All the isolates of *M. phaseolina* formed black colonies on potato-dextrose agar medium, and grow profusely from 15 to 40°C. However, the optimum

growth occurs at 30-35°C. Some of the isolates can be identified on the basis of morphological characteristics and their thermophilic nature (Satto *et al.* 1999). The sclerotia shape in most cases is irregular except for a few which are round to elongate (Mandal *et al.* 1998). The mycelium of *M. phaseolina* is septate, 1.5-2.5 μ wide, hyaline at first, turning to honey or black colour with edge. Fructification consists of globose or sub-globose pycnidium, which is formed only on infected plants and consists of 3-4 layers of blackish-brown, thin-walled angular cells and sclerotia. Pycnidia can be detected in epidermis and sclerotia in ray cells and soft tissues of the xylem and phloem. Under laboratory conditions sclerotium is hyaline to light brown in colour measuring 89 μ in diameter, whereas in soil it measures from 50-120 μ in diameter (Upadhyay *et al.* 2002).

M. phaseolina isolates from a single species differed in pathogenicity to multiple cultivars of that species in the cases of cotton (*Gossypium arboreum* L., *Gossypium hirsutum* L., *Gossypium herbaceum* L.) (Suleman and Patil 1966) and jute (*Corchorus capsularis* L., and *Corchorus olitorius* L.) (Ahmed and Ahmed 1969), leading the authors to conclude that there were distinct physiological races of *M. phaseolina*. Dhingra and Sinclair (1973b) reported that nine isolates collected from soybean across the mid-western United States varied in cultural characteristics and virulence to soybean.

M. phaseolina engenders a major problem for chickpea, which infect the roots from seedling to flowering stage at temperature range of 25-35°C and moisture stress condition in different parts of the world (Anonymous 1985). The charcoal rot disease develops as a result of series of events involved in pathogenesis, right from germination of sclerotia on root surface to visible symptoms (Ammon *et al.* 1974, 1975). Root exudates provide stimulus to

sclerotia to germinate, the developing mycelium forms appresorium on the root surface and penetrate the epidermis between the cells or enter through the cracks in surface layer of root. Appearance of reddish brown lesion on hypocotyl region is the first visible symptom of the disease, which later on turns ash gray to black colour and at disease severity wilting of the plant have been found due to complex pathogenesis (Meyer *et al.* 1974). Moreover, the fungus produces toxins (Dhingra and Sinclair 1974) and enzymes *in vitro* and *in vivo* those are responsible for disease development. Variation in morphology and virulence among the isolates of *M. phaseolina* was reported in soybean, common bean, and other crops (Dhingra and Sinclair 1972; Echavez-Badel and Perdomo 1991). However, efforts to gratify subspecies of *M. phaseolina* was based on size of micro-sclerotia, culture characteristics, changes in soil population in response to rotation, and difference in pathogenicity which usually failed because of the extreme variability within the species or difficulties in quantifying characteristics (Dhingra and Sinclair 1973b; Pearson *et al.* 1986; Cloud and Rupe 1991).

In the broader perspective due to lack of morphological variability within *M. phaseolina*, several investigators have only focused on the variation in pathogenic ability of the isolates collected from at least two host species (Chandra and Tandon 1965; Mendes *et al.* 1971; Chan and Sackston 1973; Khan *et al.* 1976; Byadgi and Hegde 1985; Pearson *et al.* 1987a). Evidence from crop rotation and intercropping suggested that soil populations of *M. phaseolina* comprise a range of host preferences (Vasudaeva and Ashraf 1939; Vasudeva 1941; Alabouvette and Marty 1977; Francl *et al.* 1988; Singh *et al.* 1990). For example, Francl *et al.* (1988) reported that when soybean, cotton and sorghum were grown in rotation, maximizing the intervals between the soybean crops minimized *M. phaseolina* populations. Since all crops included in these studies

were susceptible to *M. phaseolina* it is quite plausible that isolates may vary in host preference, with only a portion of soil population posing a threat to any single component of the particular rotation or inter-cropping system.

Insufficient morphological variability within the genus has led some workers to partition this organism based on cultural characteristics (Reichert and Hellinger 1947). Investigators have differentiated strains of this fungus based on the ability to utilize nitrate as a nitrogen source (Correll *et al.* 1986; Correll *et al.* 1987; Larkin *et al.* 1988; Bayman and Cotty 1989). Nitrate uptake does not appear to occur without nitrate metabolism (Pateman and Kinghorn 1976). The metabolic assimilation of nitrate is by the reduction to nitrite via nitrate reductase. Nitrite is then reduced to ammonia (Pateman and Kinghorn 1976). Fungi unable to use nitrate cannot synthesize nitrate reductase (Whitaker 1976). Enzyme production in *M. phaseolina* is complex, requiring regulatory structural and co-factor producing genes for the production and assembly of nitrate reductase (Marzluf 1981). Mutations within nitrate reductase structural genes result in qualitative changes within the enzyme, whereas mutations within regulatory genes alter enzyme quantity (Cove 1979).

Chlorate is an analog of nitrate, differentiation of fungal strains is based on the reduction of chlorate to chlorite by the nitrate reductase pathway that can result in chlorate toxicity (Arst and Cove 1969). Chlorite restricts growth when the nitrate reductase pathway is functional. In many fungi, sectors with unrestricted growth can develop from restricted colonies on media containing chlorate. Thus, chlorate-utilizing isolates were classified as chlorate sensitive, whereas those isolates unable to metabolize chlorate were classified as chlorate resistant (Pearson *et al.* 1986). Since chlorate is a nitrate analog,

Pearson *et al.* (1987) hypothesized that chlorate utilization might be related to nitrate utilization. In a study of more than 200 fungal isolates from 13 states they found that isolates from maize (*Zea mays* L.) were generally chlorate resistant, whereas those from soybean were generally chlorate sensitive. This result was corroborated by Cloud and Rupe (1991). However, Zazzerini and Tossi (1989) reported that *M. phaseolina* isolates from four host species varied in their chlorate utilization phenotype, irrespective of their original host, concluding that there was no evidence for host specialization within *M. phaseolina*. Further evidence for host preference was provided by Pearson *et al.* (1986, 1987a, 1987b) who demonstrated that *M. phaseolina* differed in growth on a minimal medium amended with potassium chlorate.

Unrestricted growth in these sectors results from the inactivity of one or more of the five enzymes in the nitrate reductase pathaway (Pateman and Kinghorn 1976). Such isolates are designated nitrogen mutants. These nitrogen mutants have been used to identify vegetative compatibility groups within several species of plant pathogenic fungi. However, media containing chlorate have been used to differentiate isolates on the basis of their growth morphology (Pearson *et al.* 1986; Pearson *et al.* 1987; Cloud and Rupe 1991). Chromogenicity, sporulation ability, and pycnidial size are also known to vary greatly (Crall 1948; Dhingra and Sinclair 1978; Pearson 1982). Traits with less variability would be more useful when trying to group the isolates.

II. Molecular methods for the characterization and identification of Fungi

The ability to accurately identify an organism is fundamental to all aspects of fungal diagnostics and epidemiology whether this is in the field of plant pathology, medical science, environmental studies or biological control. In plant pathology, early identification of the causative agent of disease is

paramount in order to recognize the pathogen, and implement regulations involving control and quarantine. In recent years there has been vast progress in the development of molecular biological tools and technologies. These have been increasingly applied to the study of fungal plant pathogens (Nazar *et al.* 1991; Lee *et al.* 1993; Bridge *et al.* 1998; Jana *et al.* 2005a; Purkayastha *et al.* 2006; Das *et al.* 2006).

II. 1. PCR-Based Methods

PCR-based methods offer many new tools that are directly applicable to fungal systematics at the species level. These tools can be used to delimit and to determine relationships among species, either by direct comparison or through phylogenetic analysis. PCR-based methods have given a greater insight into molecular variability within fungi and have highlighted the need to consider carefully sampling strategies and sample sizes, prior to making taxonomic decisions. This insight has also shown that molecular variability is not constant within different fungal species, and levels of both homo- and heterogeneity will vary depending upon the species studied. Perhaps surprisingly, the introduction of PCR-based techniques has not led to a widespread revision of fungal species names and concepts, and in many cases existing species concepts have been reinforced. However, the wide range of molecular heterogeneity found in some species has led to the suggestion that they may be many more "cryptic" and underscribed species within existing collections. Where PCR-based methods will have a very significant impact in the study of the 83% of known species that do not grow in culture and the hope is that these techniques may, in future, provide many answers to basic questions in systematics and biodiversity that are currently unanswered.

The introduction of PCR-based methods has significantly increased the level of activity in fungal systematics. The simplicity of the techniques, coupled with the general use of particular regions of the genome, has resulted in many important advances in our understanding of taxonomic groupings as well as the evolutionary histories and functional properties associated with them. From the mid-1980s molecular studies have been utilized in systematic to resolve problems posed by the limitation of morphological characters or in cases where morphological characters are in conflict, ambiguous, or missing (Hills 1987; Bruns *et al.* 1991; Hibbett 1992). Hawksworth *et al.* (1995) stated that there were 72,000 accepted fungal species, and that this number was growing rapidly; only 17% of these were represented in culture collections. One potential application of PCR-based techniques in their utility in situations where the sample size is smaller, and so technique developed for dried specimens and environmental samples provide opportunities to obtain material from reference collections is now a relatively straight forward procedure (Taylor and Swann, 1994; Savoliainen *et al.* 1995) and provides many opportunities for examining both current and earlier species concepts and variability.

PCR provides a simple and ingenious method to exponentially amplify specific DNA by *in vitro* DNA synthesis. This enzymatic reaction is so sensitive that a single DNA molecule can be amplified from the complex mixture of the genomic sequences that can generate large quantities of target DNA, and any nucleic acid sequences can be cloned, analyzed or modified. The technique is now considered to be indispensable in the field of modern mycology and fungal taxonomy. The PCR technique allows rapid and selective identification and/or detection of microorganisms in different matrices by amplifying specific gene fragments Southern blotting and

hybridization with a specific probe can also be carried out (Sandhu *et al.* 1995: Hendolin *et al.* 2000; Loffler *et al.* 2000). Attempts have been made to increase the specificity of PCR reactions using other methods, including post-PCR hybridization (Sandhu *et al.* 1995), PCR-ELISA reactions (Grimm and Geisen 1998; Schnerr *et al.*, 2001), RFLP analysis of the PCR products (Yamagishi *et al.* 1999), denaturing gradient gel electrophoresis (DGGE) (Cocolin *et al.* 2001), florescent capillary electrophoresis (Turenne *et al.* 1999), or nested PCR, where one set of primers is used to amplify DNA fragments from target DNA, and a second set of primers complementary to an internal sequence of the product of the first PCR reaction is used to score and confirm the results (Ibeas 1997). In case of closely related species, single nucloetide differences can be visualized by using single strand conformation polymorphism (SSCP) analysis (Kumeda and Asao 1996), heteroduplex mobility assay (Olicio *et al.* 1999), heteroduplex panel analysis (Kumeda and Asao 2001), or by sequence analysis (Cappa and Cocconcelli 2001).

Any DNA or RNA sequence that is specific for a particular organism can be used for PCR detection of that organism. The application of PCR to the study of phytopathogenic fungi has so far resided in two main areas, diagnosis and detection and the identification of variation. One of the attributes of PCR, *i.e.*, the requirement for very little template DNA, makes its use in the detection of foreign agents in host tissues very attractive. The sensitivity, speed, and versatility of PCR are primary factors in its wide acceptance in plant pathology as well as many other fields of biology. In its impact on basic and applied research, PCR is unsurpassed. It is adaptable to many experimental objectives, and it is used with a wide range of starting material, including purified nucleic acids, intact cells or tissues, or complex environmental samples. As PCR methods for detection of pathogens become

available, more research will focus on using these tools to study pathogen populations, biology, and ecology, variability, and host-pathogen interactions. The development of PCR technology relies on three fundamental steps: 1) the selection of a specific target region of DNA to identify; 2) extraction of total DNA from the sample; 3) a method to detect the presence of the target DNA/RNA region in the sample.

II. 2. Molecular targets used for the detection of Fungi

The choice of molecular target depends on the aim to be achieved. For fungal detection, usually the conserved regions the rRNA gene cluster are targeted. Other targets could also be used, including genes of the chitin synthase gene (Jordan 1994), ergosterol biosynthesis (Morace *et al.* 1997), and translation elongation factor genes (Vaitilingom *et al.* 1998). For detection of a single genus or species, the markers should be designed on the targeted regions like; more variable regions of the genome, *e.g.* spacer regions of the rRNA gene cluster, or Sequence characterized amplified region (SCAR). For the detection of mycotoxin producing fungi, sequences of the mycotoxin biosynthetic genes are the best targets. In the following, the targets used for molecular detection of fungi are dealt with, with special emphasis on mycotoxin producing fungi.

II. 3. Ribosomal DNA Gene Cluster

The DNA sequences that encode for RNAs have been extensively used to study the taxonomic relationships and genetic variations in fungi (*e.g.*, Bruns *et al.* 1992; Hibbert 1992). The ribosomal RNA gene cluster is found both in nuclei and mitochondria, and consists of both highly conserved and variable regions (White *et al.* 1990). The fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. The conserved

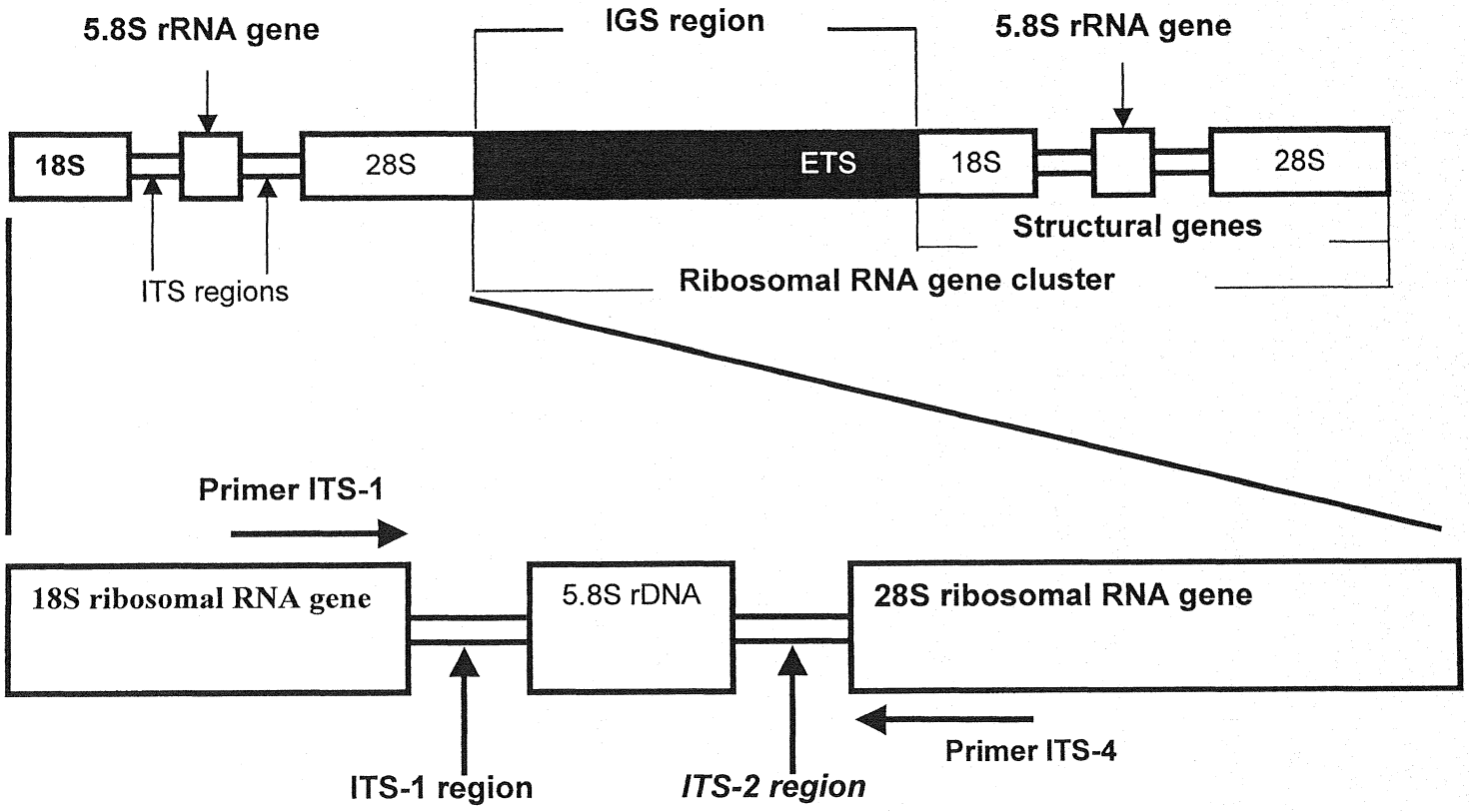

Fig. 2.1. General physical map of rDNA gene cluster in fungal genome: The complete repeat unit is represented with genes location and spacer regions. ITS – Internal transcribed spacers, IGS – Intergenic spacers, primers ITS-1 and ITS-4 are showing the specific binding position.

sequences found in the large subunit and small subunit genes have been exploited to study the relationships among distantly related fungi (*e.g.*, Gaudet *et al.* 1989; Bowman *et al.* 1992; Bruns *et al.* 1992; Bridge *et al.* 2003). The spacer regions between the subunits, called the internal transcribed spacers (ITS), and between the gene clusters, called the intergenic spacers (IGS), are considerably more variable than subunit.

Diagram of rDNA cluster (Fig. 2.1) includes three rRNA genes: the small nuclear 18S and the large nuclear 28S genes. In one unit, the genes are separated by two internal transcribed spacers (ITS1 and ITS2), and two rDNA units are separated by the intergenic spacer (IGS). These genes have been used widely on studies on the relationships among species within a single genus or among interspecific populations (Buchko and Klassen 1990; Spreadbury *et al.* 1990; Nazar *et al.* 1991; Taylor and White 1991; Anderson and Stasovki 1992; Baura *et al.* 1992; Lee and Taylor 1992; Kim 1992; O'Donnell 1992; Molina *et al.* 1993; Erland *et al.* 1994; Li *et al.* 1994; Buscot *et al.* 1996; Arora *et al.* 1996). The last rRNA gene (5S) may or may not be within the repeated unit. Numerous sequence data are now available and allow the determination of primer sequences for the PCR amplification of different parts of the nuclear rDNAs (White *et al.* 1990). There are now many examples of the use of either RFLP or sequence differences in different spacer regions for discriminating between closely related species within a fungal genus.

II. 4. 18S rRNA Gene as Target

There are several reports where 18S rDNA sequences have been used for the detection and identification of fungi. Kappe *et al.* (1996), Smit *et al.* (1999), and Borneman and Hartin (2000) developed primer pairs based on this region for the detection of wide range of fungi. Meyer (2002) also developed a primer

pair and at *Taq*man probe based on 18S rDNA sequences, which was used successfully for real-time PCR detection of fungi in black pepper, red pepper, corn, and cereal samples. Makimura *et al.* (1994) developed a PCR detection system based on 18S rRNA gene sequences for detection of *Aspergillus* and *Penicillium* species. Cappa and Cocconcelli (2001) developed an 18S rRNA-based assay for the detection of fungi in food samples.

II. 5. 28S rRNA Gene as Target

Although the DNA coding for the large (28S) ribosomal rRNA subunit is relatively conserved and is more commonly used for work at higher taxonomic levels, certain portions of it, particularly the eukaryotic D1 and D2 divergent domains near the 5′ end are variable enough to detect species-specific differences. The D1-D2 region has extensively been used for phylogenetic studies of *Aspergillus*, *Penicillium*, and yeast as well as other fungi (Peterson 2000; Rigo *et al.* 2001). Universal 28S rDNA-based primers were developed by Sandhu *et al.* (1995).

II. 6. Intergenic Transcribed Spacer

The ITS region has been most frequently used as target for species-specific detection of fungi. The ITS region consists of two non-coding variable regions that are located within the rDNA repeats between the highly conserved small subunit, the 5.8S subunit, and the large subunit rRNA genes. The ITS region is a particularly useful area for molecular characterization studies in fungi for four main reasons: (i) the ITS region is relatively short (600-800bp) and can be easily amplified by PCR using universal single primer pairs that are complimentary to conserved regions within the rRNA subunit genes (White *et al.* 1990), (ii) the multicopy nature of the rDNA repeat makes the ITS easy to amplify from small, dilute or highly degraded DNA samples (Gardes and

Bruns 1993), (iii) the ITS region may be highly variable among morphologically distinct species (Gardes and Bruns 1991; Gardes *et al.* 1991; Chen *et al.* 1992; Lee and Taylor 1992; Gardes and Bruns 1993), ITS-generated RFLPs restriction data can be used to estimate genetic distances and provide characters for systematic and phylogenetic analysis (Bruns *et al.* 1991) and (iv) PCR generated ITS species-specific probes can be produced quickly, without the need to produce a chromosomal library (*e.g.* Sreenivasaprasad *et al.* 1996) and many researchers have selected sequences from the ITS region to develop species-specific probes because the sequences occur in multiple copies and tend to be similar within and variable between fungal species.

This region was targeted for the detection of spoilage yeast including *Zygosaccharomyces* spp. and *Torulaspora delbrueckii* (Sancho *et al.* 2000), *Saccharomyces* (Arlorio *et al.* 1999), *Alternaria* spp. (Zur *et al.* 1999), *Penicillia* (Pederson *et al.* 1997; Boysen *et al.* 2000), and *Fusarium avenaceum* (Schilling 1995). Grimm and Geisen (1998) developed ITS-based primer pairs for the detection of fumonisin producing *Fusarium* species. Hendolin *et al.* (2000) developed a PCR technique coupled with multiplex liquid hybridization based on ITS specific primers for the detection of a number of fungi in clinical specimens. PCR-amplified rRNA ITS sequences have been used for the characterization, identification and detection of *Verticillium albo-atrum* and *V. dahliae* (Nazar *et al.* 1991). In this study the identification of distinct clusters of non-homologous nucloetides in both the ITS1 and ITS2 regions enabled the design of specific primers that provides a reliable identification/detection method of these two important plant pathogens (Nazar *et al.* 1991). The same principle was used by Moukhamedov *et al.* (1993) who used sequences from amplified regions of the 5.8-28S ITS regions to differentiate *V. tricorpus* from other species of *Verticillium*. The genus *Rhizoctonia* consists of a taxonomically

diverse group of species that differ in many significant features, including their sexual and asexual stages (Sneh *et al.* 1996) within the important phytopathological species *R. solani,* further intra-specific groups have been designated on the basis of anastomosis (anastomosis groups; AGs). Originally, RFLP analysis of nuclear rDNA was undertaken with probes and Southern blotting (Jabaji-Hare *et al.* 1990; Vilgalys and Gonzales 1990), and more recently PCR-amplified rRNA has been found to be useful in examining the genetic relatedness within different AGs of *R. solani* and binucleate species of *Rhizoctonia* (Kanematsu and Naito 1995 Liu *et al.* 1995; Vigalys and Cubeta 1994; Hyakumachi *et al.* 1998). These workers identified six subgroups within AG 1 and five within AG 2 on the basis of their ITS-RFLPs. Boysen *et al.* (1996) used an asymmertric PCR technique on the ITS1, ITS2 and 5.8S rDNA regions with nine AG 4 *R. solani* isolates. These data were used in a phylogenetic analysis which identified three subgroups within AG 4. Mazzola *et al.* (1996) developed species-specific primers for the detection of *R. oryzae* by comparing ITS1 and ITS2 sequences from *R. oryzae* and *R. solani* AG1, 5, 6 and 8. These primers were specific to *R. oryzae* but not to *R. solani* or binucleate species.

Edel *et al.* (1996) differentiated several strains of *F. oxysporum* at the species level by RFLP analysis of a region of ITS and a variable domain of the 28S rDNA. Recently, Schilling *et al.* (1996) evaluated sequence variation in the ITS regions of *F. avenaceum, F. culmorum* and *F. graminearum* in order to distinguish between the three species. They found that the ITS sequences of *F. culmorum* and *F. graminearum* were not polymorphic enough to allow the construction of species-specific primers; however, sufficient sequence variation was found in the ITS1 and ITS2 regions of *F. culmorum* and *F. graminearum* to distinguish them from *F. avenaceum.*

Kageyama *et al.* (1997) have used species-specific primers derived from ITS sequences to detect *Pythium ultimum* in naturally infected seedlings. Bunting *et al.* (1996) used ITS1 sequences to examine the relationship of *Magnaporthe poae* to other species in the genus that has similar growth or phytopathogenic characteristics. Poupard *et al.* (1993) used amplified ITS regions in their characterization of *Pseudocercosprella herpotrichoides* isolates. PCR-RFLP of ITS has also allowed the discrimination of *Tuber* species (Carbone and Kohn 1993), the identification of species within the *Gaeumannomuyces-Phialophora* complex (Ward and Akrofi 1994), *Sclerotinia* species (Carbone and Kohn 1993), *Cylindrocarpon heteronema* (Brown *et al.* 1993) and *Pencillium* species (Lobuglio *et al.* 1993). One further example of a genus where extensive use has been made of the ITS region of the species level is *Colletotrichum.* Sherriff *et al.* (1994) compared a range of isolates of *Colletotrichum* species on the basis of 850 bp region of the LSU and the ITS2 regions, and were able to use this information to distinguish between individual species. Further extensive species characterization has been undertaken in this genus, leading to the development of a number of species-specific primers (Sreenivasaprasad *et al.* 1996).

Recently, Kong *et al.* (2004) has studied Single-strand conformation polymorphism (SSCP) analysis to identify different species based on ITS-rDNA, which can detect single base mutations or variations (Orita *et al.* 1989; Rubio *et al.* 1996; Kong *et al.* 2000). A protocol for SSCP analysis of ribosomal DNA for separation of 29 species within the genus *Phytophthora* was reported. SSCP analysis worked well for all tested species.

At a different taxonomic level, universal primers have been developed from rDNA sequences which are specific for major groups of fungi. Gardes

and Bruns (1993) designed two taxon-selective primers (ITS1-F and ITS4-B) from the ITS region which were specific to fungi and basidiomycetes respectively. The primer ITS-4B when combined with universal ITS1 primers or with fungal-specific primer. Primers ITS1-F/ITS4-B was useful for the detection of basidiomycete ectomycorrhizae in rust infected tissues and could be used to study both the distribution of rusts on alternate hosts and ectomycorrhizal communities.

II. 7. Random Amplified Polymorphic DNA

One approach used for developing suitable species- or strains-specific probes for the detection of fugi is based on the random amplified polymorphic DNA technique (RAPD) (Williams *et al.* 1990). The RAPD is a variation of conventional PCR where one primer of arbitrary sequence is used, and the annealing temperature is low (usually 35°C). Species or strain-specific RAPD fragments are selected, sequenced, and suitable primers are devised to amplify the specific fragment in conventional PCR reactions. Such SCAR markers have been successfully used to develop species-specific probes for a number of *Fusarium* species (Chelkowski *et al.* 1999; Nicholson *et al.* 1996; Nicholson *et al.* 1998; Schilling *et al.* 1996; Young *et al.* 2001), particularly fumonisin-producing fusaria (Geisen 1998), and for *Aspergillus fumigatus* (Brandt *et al.* 1998). Murillo *et al.* (1998) developed a primer pair based on the sequence of a random genomic clone for the detection of *Fusarium moniliforme*.

The random amplified polymorphic DNA (RAPD) fingerprinting assay detect small inverted nucleotide sequence repeats throughout the genomic DNA (Welsh and McClelland 1990; Williams *et al.* 1990. In RAPD-PCR, amplification involves only single primers of arbitrary nucleotide sequence.

The principle of RAPD assays is discussed in detail by Hadrys *et al.* (1992) and Tingey and del Tufo (1993). In brief, a single primer binds to the genomic DNA on two different priming sites in an inverted orientation. Amplification between these points results in a discrete product. As each primer can be expected to amplify several discrete loci in the genome the final result is a profile of amplification products generally of varying sizes. In addition, at the primer attachment stage in the amplification the annealing temperature is kept low which also encourages a degree of primer-mismatching, and increases the potential number of amplification products. There are many advantages of this assay: (1) no prior information for DNA sequence is needed. The protocol is relatively simple and quick and only nanogram quantities of DNA are required to give a PCR product, (2) the technique is preferred when the genotypes of a large number of species, population or pathotypes has to be discriminated. RAPD markers can also be used to analyze the genotypes of fusion products and parents at different taxonomic levels, (3) this PCR-based assays is a good tool for creating genetic maps (Judelson *et al.* 1995) and has proved as an efficient method for the identification of molecular markers (Tingey and del Tufo 1993) and (4) the technique is suitable for studying population genetics and has been successfully used to differentiate among species and strains within species of plants, bacteria, animals and fungi (Williams *et al.* 1990).

RAPD-PCR assays have been used extensively to define fungal populations at specific, intraspecific, race and strain levels. In general, most studies have concentrated on intra-specific grouping, although others have been directed at the species level. Particularly in the determination of distinct intraspecific groups such as anastomosis groups in *Rhizoctonia solani* (*e.g.*, Duncans *et al.* 1993) and pathogen groups (*e.g.*, Crowhurst 1991; Levy *et al.*

1991; Guthrie *et al.* 1992; Assigbetse *et al.* 1994; Bidochka *et al.* 1994; Nicholson and Rezanoor 1994; Burmester and Wostemeyer 1994; Yates-Siilata *et al.* 1995; Bridge *et al.* 1997a; Maurer *et al.* 1997). Another application of RAPD-PCR has been in the determination of the individual strains within a particular population, some examples being toxin-producing strains in *Aspergillus flavus* (Bayman and Cotty 1993) and in strain authentication in species of *Trichoderma* (Schlick *et al.* 1994; Fujimori and Okuda 1994).

In general, most studies have concentrated in intraspecific grouping, although others have been directed at the species level. Some examples of RAPD-PCR at species level include the production of species-specific probes and primers from RAPD data for *Fusarium oxysporum* f. sp. *dianthi, Phytophora cinnamoni, Tuber magnatum* and *Glomus mosseae* (Dobrowolski and O'Brien 1993, Lanfranco *et al.* 1993, 1995; Manulis *et al.* 1994). In some RAPD-PCR studies, band patterns have been used to differentiate both within and between the individual species, as exemplified by species of *Metarhizium* and *Candida* (Lehmann *et al.* 1992; Bridge *et al.* 1997a).

Single simple repetitive primers have been designed to amplify the microsatelite regions of fungal chromosomal DNA (Meyer *et al.* 1992; Schlick *et al.* 1994; Bridge *et al.* 1997b). In most applications these primers have given been used to group fungi at intraspecific levels (*e.g.*, Bridge *et al.* 1997a). However, in some instances microsatellite-primed PCR has been used to generate species-specific patterns and one recent example of this is the work on morels by Buscot *et al.* (1996) who found considerable homogeneity from both mono and polysporic isolates of individual species.

II. 8. Similarity coefficients

The majority of dendrograms derived from fungal DNA fragments have been derived with one of three coefficients, the simple matching coefficient, Jaccard's coefficient and Nei and Li's genetic distance. The last two of these coefficients do not consider matching negative results, *i.e.*, they do not consider the absence of a particular band in two organisms as a similarity, unlike the simple matching coefficient where matching positive and negative results are considered equally (Sneath and Sokal 1973; Nei and Li 1979; Bridge 1992). The validity of considering matching negative characters has been discussed on many occasions (Sneath and Sokal 1973; Abbott *et al.* 1985) but it is perhaps worth further consideration for gel-derived data. When numerical methods are not used and gel patterns are compared by eye, the operator will use the presence and absence of bands to designate patterns. Therefore, the inclusion of matching negatives within a numerical system may be considered as representative of this, and shape coefficients such as the correlation coefficient have been used in this way in automated gel comparison software. However, in taxonomic studies matching negative characters may be acceptable when the characters themselves are relevant and comparison is meaningful. However, when bands are of unknown origin, such as in RAPD studies, then their relevance cannot be quantified and it would seem most appropriate to use coefficients that discount matching negatives.

CHAPTER III

MATERIALS AND METHODS

I. Morphological identification and characterization of *M. phaseolina*

1. Isolation and identification

2. Pathogenecity assay and Pycnidia formation

3. Preservation

II. Molecular characterization and identification of *M. phaseolina*

1. Amplification of internal transcribed spacers (ITS) region

   a. 5.8S rDNA gene amplification and RFLP.
   b. 28S rDNA amplification and RFLP.
   c. Restriction Fragment Length Polymorphism

2. Direct Sequencing of ITS region

3. Development of specific oligonucleotide primers and probe

4. Development of a PCR assay specific for *M. phaseolina*

5. Development of a hybridization assay specific for *M. phaseolina*

6. SSCP-analysis of 5.8S rDNA gene

III. Genomic fingerprinting by RAPD-PCR analysis

1. Fingerprinting by 10-mer primers (Operon kit)
2. Fingerprinting by BOXA1R primer
3. Fingerprinting by M-13 mini satellite primer
4. Data analysis

MATERIAL AND METHODS

I. 1. Isolation and identification of *M. phaseolina*

Macrophomina phaseolina (Tassi) Goid, causal agent of damping of seedling blight, collar rot, stem rot, root rot and charcoal rot of various crops plants was isolated from the roots, stem and soils of diseased bean, chickpea, corn and soybean plants. The diseased plants and the soils were collected from different sites. Infected plants were uprooted with the help of a spade and samples of the diseased plants were kept in UV sterilized polythene bags and brought under refrigerated conditions.

Pieces of diseased roots and stems (1 cm long) were washed four times with sterilized distilled water (SDW) and surface disinfected with 1% ethanol for 2-3 min and again washed twice in SDW. One cm long, root and stem segments were placed (2 piece per petri dish of 9 cm diameter) on acidified potato dextrose agar (APDA) medium. Plates were incubated at 28±2°C for six days. For purification, colonies growing out of root and stem pieces were re-inoculated on fresh APDA medium. The pure cultures were stored on APDA slants at 4°C for further study. Soil isolates of other fungi were stored in petri dishes at 4°C containing APDA media.

The isolated *M. phaseolina* colonies were identified on the basis of cultural and morphological characteristics (Gillman 1967; Schots *et al.* 1994). Some reference isolates of *M. phaseolina* isolates were obtained from the culture collection of UK-CABI and all the isolates were submitted to the repository of National Bureau of Agriculturally Important Microorganisms (NBAIM), India (Table 3.1).

Table 3.1 *M. phaseolina* isolates collected from different hosts and different locations used in this study.

Sl. No	Code No.	NBAIM Accession No.	Geographical origin	Biological origin
1	mpk1	F-1289	Himachal Pradesh	Potato
2	mpk2	F-1266	Himachal Pradesh	Potato
3	mpk3	F-1295	Ballia, Uttar Pradesh	Chickpea
4	mpk4	....	Maharastra	Sorghum
5	mpk5	F-1263	Varanasi, Uttar Pradesh	Chickpea
6	mpk6	F-1288	Varanasi, Uttar Pradesh	Sorghum
7	mpk7	F-1274	Jhansi, Uttar Pradesh	Soybean
8	mpk8	F-1286	Mau, Uttar Pradesh	Corn
9	mpk9	F-1264	Ballia, Uttar Pradesh	Soybean
10	mpk10	.....	Kerala	Soil
11	mpk11	F-1272	Varanasi, Uttar Pradesh	Chickpea
12	mpk12	F-269	Mau, Uttar Pradesh	Soil
13	mpk13	CABI-263176	Uttar Pradesh	N.A
14	mpk14	F-1278	Varanasi, Uttar Pradesh	Pea
15	mpk15	F-1285	Karnataka	Corn
16	mpk16	....	Gujarat	Sorghum
17	mpk17	F-1281	Karnataka	Chickpea
18	mpk18	F-1267	Delhi	Mung bean
19	mpk19	F-1269	Delhi	Soybean
20	mpk20	F-302	Jorhat, Assam	Sorghum
21	mpk21	F-1291	Delhi	Sorghum
22	mpk22	F-494	Chennai	Corn
23	mpk23	F-1271	Delhi	Chickpea
24	mpk24	F-1284	Andhra Pradesh	Soybean
25	mpk25	F-1294	Delhi	Pea
26	mpk26	F-1270	Madhya Pradesh	Soil
27	mpk27	F-1268	Madhya Pradesh	Soybean
28	mpk28	....	Tamil Nadu	N.A
29	mpk29	CABI-277878	N.A	N.A
30	mpk30	F-496	Madhya Pradesh	Ground nut
31	mpk31	F-1276	Solapur, Maharastra	Sorghum
32	mpk32	F-495	Varanasi, Uttar Pradesh	French bean
33	mpk33	F-300	Jorhat, Assam	Chickpea
34	mpk34	F-1273	Karnataka	Soybean

35	mpk35	F-1262	Varanasi, Uttar Pradesh	Soybean
36	mpk36	F-1261	Jhansi, Uttar Pradesh	Soil
37	mpk37	F-301	Uttar Pradesh	Soybean
38	mpk38	F-1297	Varanasi, Uttar Pradesh	Chickpea
39	mpk39	F-1287	Ballia, Uttar Pradesh	Sorghum
40	mpk40	F-1296	Mau, Uttar Pradesh	Chickpea
41	mpk41	F-1277	Gulberga, Maharastra	Sorghum
42	mpk42	F-1275	Andhra Pradesh	Sorghum
43	mpk43	F-1280	Uttar Pradesh	Corn
44	mpk44	....	Rajasthan	Sorghum
45	mpk45	F-1292	Varanasi, Uttar Pradesh	Sorghum
46	mpk46	F-1290	Andhra Pradesh	Corn
47	mpk47	F-1279	Chennai	Soybean
48	mpk48	F-1265	Varanasi, Uttar Pradesh	Chickpea
49	mpk49	F-1283	Varanasi, Uttar Pradesh	Sunflower
50	mpk50	F-1282	Jhansi, Uttar Pradesh	Corn

I. 2. a.Pathogenecity assay

The occurrence of disease in their respective susceptible host plants (Sorghum, chickpea and soybean) was determined in pathogenecity assay under greenhouse conditions. For pathogenecity, 50 isolates were used. Toothpick inocula method was used for inoculation of the pathogen on host plants (Edmunds 1964). The plants were inoculated 14-23 days after sowing. For each experiment, one set of uninoculated control plants were used. Toothpicks were inserted in to the plant stem with no prior wounding. Incubation period that followed inoculation, ranged between 6-14 days, and incubation temperature was maintained in the range of 28-30°C. In this experiment, 20 plants per isolate were used. For data analysis, the number of symptomatic plants was only taken in account. In the experiment with bean, chickpea, corn and soybean plant, only those plants that were completely wilted or dead were considered as diseased.

I. 2. b. Pycnidia formation

The ability of *M. phaseolina* isolates to form pycnidia on leaf tissues in their respective host plants as investigated as described by Mihail (1992). Inoculation method was same as above; each plant was inoculated at its flowering stage. Incubation period in all the hosts (*i.e.*, Sorghum, Chickpea and Soybean) ranged from 6-12 days. Following incubation, approximately in which pycnidium was removed form the leaf tissue and crushed on a microscope glass slide and the presence of typical pycnidio-spores were confirmed.

I. 2. c. Data analysis

Data analysis for pathogenecity and pycnidia formation was done following the procedure of Mihail and Taylor (1995). For pathogenecity the sum of all symptomatic plants was determined for each of the four host species. Similarity for each host in the pycnidia formation test, the sum of leaf pieces in the experiment with pycnidium was determined I (maximum=10). If the sum for pathogenecity or pycnidia formation was zero, one or greater than one, the corresponding phenotype code for that type of experiment was considered zero, two or three respectively. This coding system was adopted to give greater weight to the difference between pathogenecity and non pathogenecity than to degree pathogenecity similarity, the difference in frequency of pycnidium formation. All the experiments were repeated twice and data were subjected to standard deviation.

I. 3. a. Lyophilization of *M. phaseolina* isolates

A spore suspension was prepared in a 10% (w/v) skimmed milk solution pre-sterilized by autoclaving at 114°C for 10 min. With a Pasteur pipette 0.2mL (approx.) was added as a suspension to each sterile ampoule. Each ampoule was covered with a sterile lint cap. The ampoules were loaded onto the circular

manifold and place in a freezer, when frozen place into a dry ice bucket then attached to the freeze drier chamber manifold. The ampoules were left attached to the chamber and evacuated for a 5h. Air was admitted into the freeze-drier chamber and the ampoules removed. The ampoules were plugged with sterile cotton wool compressed to 10mm in depth, 10mm (approx.).

The plugged ampoule was constricted 10mm above the cotton plug using the air/gas torch. The constricted ampoules were placed on the manifold accessory of the freeze-drier and evacuated. The ampoules were sealed at the point of constriction after a 16h (over night) drying process using a twinjet burner under vacuum (Vacuum and Industrial Products). At this point the moisture content was 1-2% by dry weight.

I. 3. b. Recovery and testing of Lyophilized cultures

An ampoule was scored at the centre of the cotton wool plug using a glasscutter. The tip of a glass rod was heated in a Bunsen burner until red-hot and applied firmly to the score. The heat cracked the tube around the score line. The ampoule was Snap-opened and the cotton plug removed. With a Pasteur pipette 2-4 drops of sterile distilled water was added and the cotton plug replaced leaving for 30 min to rehydrate the suspension. This suspension was then inoculated onto a suitable growth medium and incubated under appropriate conditions.

II. Molecular characterization and identification of *M. phaseolina*

II. 1. Extraction and quantification of DNA

The fungus was grown in 150 mL of malt extract broth and gyrated at 125 rpm for 5-6 days at 28±2°C. The mycelia were harvested by filtration through a double layered cheese cloth and freeze dried under vacuum. Total genomic DNA was extracted, as described by Lee and Taylor (1992), with some modifications. Approximately 200 gm of lyophilized mycelia was taken into 1.5 mL tube, crushed with micro-pestle mixed with 300 µL of lysis solution (50mM Tris-HCl, pH 7.8, 50mM Na_2-EDTA, 3% SDS) to which freshly prepared 1% 2-mercaptoethanol was added and incubated at 65 °C for 4 h. Proteinase K (600µg mL^{-1}) was added and further incubated at 35°C for 1 h. The genomic DNA was purified by phenol-chloroform method and precipitated with ethanol (*Appendix*-I). The pure and quantified DNA samples were stored at 4°C till further use.

II. 1.a. PCR amplification of 5.8S rDNA gene cluster

The rDNA gene cluster, consisting of ITS-1, 5.8S rDNA and ITS-2, was amplified with primers homologous to conserved sequences within the small subunit (SSU) rDNA gene. The ITS primers used were ITS-1 and ITS-4 (White *et al.* 1990) (Table 3.2). PCR was performed in a total volume of 50 µL containing 5 µL of 10X PCR buffer (100mM, Tris-HCl, pH 8.3, 15mM MgCl2, 250mM KCl), 1U *Taq* DNA polymerase (Bangalore Genei, India), 160 µM dNTP mixture, 50 pmol of each ITS-1 and ITS-4 primers, and 50 ng genomic DNA in SDW. PCR was carried out for 35 cycles of denaturation at 95°C for 1min, annealing at 50°C for 30 sec and extension at 72°C for 1min 20 sec, with a final extension step at 72°C for 10 min, using Techne, TC-412 thermocycler.

Table 3.2 List of primers used for amplification of genomic DNA

Sl. No	Primers	Sequence (5'-3')
1	ITS-1	TCCGTAGGTGAACCTGCGG
2	ITS-4	TCCTCCGCTTATTGATATGC
3	P3	TCCGTAGGTGAACCTGCGG
4	BOXA1R	CTACGGCAAGGCGACGCTGACG
5	M13 mini-satellite	GAGGGTGGCGGTTCT
6	URP9F	ATGTGTGCGATCAGTTGCTG
7	URP P1F	ATCCAAGGTCCGAGACAACC

II. 1. b. PCR amplification of 28S rRNA gene cluster

PCR amplification of a part of 28S rRNA gene cluster was carried out as described by Guadet *et al.* (1989). Amplification was carried out in 50 µL reaction mixture with 50 pmoles of specific primers ITS-1and P3 (Guadet *et al.* 1989). The reaction mixture consist of 5 µl of 10X PCR assay buffer (10mM Tris HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton-X 100), 1.2 µL of 50 pmol primers, 0.2 µL of 25 mM dNTPs, 0.4 µL of 3U/µL Taq polymerase, 1.5 µL (20-50ng) of template DNA, and 46.2 µL of sterile distilled water. Amplification reaction was performed in BioRad Dyad thermocycler using block control, initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 50 sec, extension at 72°C for 1min 20 sec, followed by final extension at 72°C for 10 min.

II. 1. c. Restriction Fragment Length Polymorphism analysis

The amplified rDNA fragment was used for RFLP analysis using 10 restriction enzymes (Table 3.4). A volume of 20 µL reaction mixture containing 2 µL of 10X buffer, 0.8µL restriction enzyme (10U/µL), 14 µL amplified DNA and 3.2 µL sterile distilled water was prepared for each sample and incubated overnight at 37°C in a water bath. The restriction digests were separated by electrophoresis in 2% agarose

gel for 6 h at 6 Vcm^{-1} in 1X TBE buffer, stained with Et-Br and visualized under UV transilluminator.

Table 3.3 Master mixtures for 1X- PCR

Reaction mixture	**ITS**		**Specific primers**
Primer sets	5.8S (ITS 1 and ITS 4)	28S (ITS 1 and P3)	MPKF1 and MPKR1
Genomic DNA	50 ng	50 ng	25-35 ng
P-1	50 pmol	50 pmol	5 pmol
P-2	50 pmol	50 pmol	5 pmol
dNTP mix	0.2 mM	0.2 mM	0.2
10x PCR-Buffer	5 µL	5 µL	2 µL
Enzyme	1 U	1 U	0.4 U
Deionized water	To 50 µL	To 50 µL	To 20 µL

II. 2. Direct sequencing of ITS region

Eight isolates of *M. phaseolina* collected from different host and diverse ecological conditions were selected for sequencing of ITS region. For direct sequencing of PCR products, the amplified product was electrophoresed on 1% agarose gel and the fragment was extracted and purified using the Prepagene kit (BioRad). Sequencing was carried out on an ABI automated DNA sequencer, using cycle sequencing with the ABI Big Dye termination cycle sequencing ready reaction kit following the protocol recommended by the manufacturer. The resulting ITS sequences were analyzed for homologies with sequences deposited in the GenBank and EMBL databases.

II. 3. Development of specific oligonucleotide primers and probe

The eight sequences from *M. phaseolina* isolates and other two reference sequences retrieved from EMBL and GenBank databases (Table 4.2) were aligned using the CLUSTAL X (version 1.7) algorithm program .

Table 3.4 Restriction enzymes used for RFLP analysis

Sl. No	Restriction enzyme	Recognition site
1	*Eco*R I	G▼AATTC
2	*Sau* 3A I	▼GATC
3	*Alu* I	AG▼CT
4	*Cla* I	AT▼CGAT
5	*Hap* II	C▼CGG
6	*Msp* I	C▼CGG
7	*Taq* I	T▼CGA
8	*Apa* I	GGGCC▼C
9	*Hind* III	A▼AGCTT
10	*Sac* I	GAGGCT▼C

The sequences were visually checked for regions having homologies among isolates of *M. phaseolina* but not among other fungi. The regions, which were conserved among the isolates and specific for *Macrophomina,* were selected to design species-specific oligonucleotide. An oligonucleotide probe (MpKH1) was designed from the conserved region, adjacent to the 5.8S gene. Two primers, forward and reverse were designed using Primer3 online software with default options. The forward (MpKFI) and reverse (MpKRI) primers (Table 4.3) were evaluated separately to yield a product in the range of 300 to 400 bp. The theoretical specificity of the primer set was checked with the sequences from the other fungi in the GenBank by using BLASTn analysis. The parameters such as percentage of G+C content, absence of self-complementarity in oligonucleotides and complimentarity between the primers were analyzed using the program GeneRunner (Hastings Software, USA)(*Appendix*-I). The oligonucleotides were custom synthesized by Bangalore Genei, India.

II. 4. Development of a PCR assay specific for *M. phaseolina*

The specific primers MpKFI (5'-CCGCCAGAGGACTATCAAAC-3') and MpKRI (5'-CGTCCGAAGCGAGGTGTATT-3'), designed during the course of this study were used for amplification. PCR protocol was standardized and

amplification was performed in 20 µL by mixing 2 µL of 10X PCR buffer, 0.2 mM dNTPs, 5 pmol of each primer, 0.4U of *Taq* DNA polymerase and 35 ng of template DNA. The PCR reaction was carried out for 25 cycles of denaturation at 95°C for 30 sec, followed by annealing at 56°C for 1 min, extension at 72°C for 2 min and the final extension at 72°C for 10 min.

II. 5. Development of a hybridization assay specific for *M. phaseolina*

The oligonucleotide probe MpKH1 (5'-GCTCTGCTTGGTATTGGGC-3') was non-radioactively labeled by using DIG DNA Labeling and Detection Kit (Roche Applied Science, Germany), following manufacturer's instructions. The probe was tested with 50 isolates of *M. phaseolina* to check the specificity of the probe. PCR amplified ITS product from representative strains of some common soil fungi, bacteria and actinomycetes (Table 4.3) and two strains of *M. phaseolina* (as control) were used as target in a dot blot assay to confirm the specificity of the probe. PCR products were manually blotted onto positively charged nylon membrane (Roche Diagnostics, USA). Initially, variable quantities of PCR products were blotted to optimize the concentration of target DNA for hybridization. Finally 5 µL of PCR products were blotted irrespective of their concentrations. Membranes were incubated for 15 min in a denaturation solution (0.5 M NaOH, 1.5 M NaCl), 10 min in a neutralization solution (0.5 M Tris-HCl, pH 7.5, containing 3 M NaCl), and 2 min in 10X SSC and dried at 25±2°C for 15 min. The DNA was fixed to the membrane by baking at 120°C for 30 min. To optimize the hybridization procedure, conditions like temperature for hybridization and washing, labeling method and the concentration of the probe, salt concentration of the washing buffer was determined as described elsewhere (Edel *et al.* 2000). Visual detection of the hybridized DIG-labeled probes was carried out using colorimetric substrates

(NBT/BCIP). Dot blot hybridization was performed at least three times under the conditions optimized for specific detection of *M. phaseolina*.

II.6. SSCP- Analysis of 5.8S rDNA gene:

All *M. phaseolina* isolates were subjected for the amplification of 5.8S gene by using the species specific primer set (MpKF1 and MpKR1) developed in this study (Table 4.3). DNA amplification was carried out under the optimized PCR conditions as mentioned above and SSCP analysis of the PCR products was performed as described previously. One microlitre of individual PCR products was mixed with 9 mL of the denaturing buffer (95% formamide, 20 mM EDTA and 0.05% bromophenol blue). After a brief spin, mixtures were heated at 96°C for 10 min then chilled on ice. Five microlitres of each mixture was loaded on a 12% acrylamide: bis-acrylamide (37.5:1) non-denaturing gel cast using Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA) (*Appendix-I*). Denatured PCR products were eletrophoresed in pre-chilled 1X TBE buffer, at 200 V for 2 h at room temperature.

After electrophoresis, polyacrylamide gels were peeled from the glass plate and soaked in 50 mL (for two gels) of 10% ethanol for 10 min, and placed in the same amount of 1% nitric acid for 3 min. After two brief washes with 100 mlL SDW, gels were stained in 50 ml of 2 ppm Silver nitrate for 20 min then rinsed three times in 200 mL SDW. Gels were developed by briefly rinsing in 30 mL of 1 ppm formaldehyde in 3% Sodium carbonate until desired band intensity was reached. The stain was fixed in 1% acetic acid once the SSCP patterns were visible. Images were captured for documentation and comparison analysis.

III. 1. Random Amplified Polymorphic DNA (RAPD) analysis

M. phaseolina isolates were subjected for PCR amplification for the analysis of genetic diversity by RAPD method using 13 randomly selected 10-mer random oligonucleotide primers from Operon kit (Table 3.5).

PCR-RAPD amplification reactions were carried out using the same DNA as used for ITS regions. The reaction mixture consisted of 0.2 mM of each dNTPs, 50 pmol primer, 1X PCR buffer, 10 mM Tris HCl (pH 8.3) , 50 mM KCl , 1.5 mM $MgCl_2$ and 2U of *Taq* DNA polymerase (Bangalore Genei; India). DNA amplifications were performed in BioRad Dyad thermocycler with one cycle of initial denaturation (94°C, 1 min, annealing (35°C, 2 min) and extension (72°C, 2 min) with a final extension at 72°C for 5 min.

Table 3.5 List of 10-mer random primers used in this study

Primers	Seq 5′ to 3′
OPA-15	TTCCGAACCC
OPA-11	CAATCGCCGT
OPB-08	GTGAGCTAGG
OPV-17	GACCGCTTGT
OPA-10	GTGATCGCAG
OPC-10	TGTCTGGGTG
OPB-06	TGCTCTGCCC
OPB-07	GGTGACGCAG
OPD-11	AGCGCCATTG
OPB-17	AGGGAACGAG
OPA-09	GGGTAACGCC
OPE-01	ACCGCGAAGG
Oligo-9	GTGATCGCA

III. 2. Rep -PCR

rep-PCR fingerprints were obtained for same DNA samples by using single short repetitive primer BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) (Versalovic *et al.* 1998). PCR was performed in a total volume of 25 µL containing

2.5 µL of 10X PCR buffer (100 mM, Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl), 1U *Taq* DNA polymerase (Bangalore Genei, India), 160 µM dNTP mixture, 50 pmol of primer, and 50 ng genomic DNA in SDW. PCR program was initiated by denaturation at 95°C for 7 min, followed by 30 cycles consisting of 94°C for 1 min, 54°C for 1 min and 65°C for 8 min. the reaction was terminated with an extension step consisting of 72°C for 10 min.

III. 3. URP- PCR

PCR fingerprints were obtained for same DNA samples by using Universal Rice Primers (URP) URP9F and URP P1F (Table 3. 2). PCR was performed in a total volume of 25 µL containing 2.5 µL of 10X PCR buffer (100 mM, Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl), 1U *Taq* DNA polymerase (Bangalore Genei, India), 160 µM dNTP mixture, 50 pmol of primer, and 50 ng genomic DNA in SDW. PCR program was initiated by denaturation at 95°C for 7 min, followed by 30 cycles consisting of 94°C for 1 min, 54°C for 1 min and 65°C for 8 min, the reaction was terminated with an extension step consisting of 72°C for 10 min.

III. 4. M13 mini satellite -PCR

PCR amplification reactions were carried out using the same DNA as used for the above reactions. The, 25 µL reaction mixture contains 25-30 ng genomic DNA, 0.2 mM of each dNTPs, 50 pmol primer, 1X PCR buffer, (10 mM Tris-HCl (pH 8.3), 50 mM KCL, 1.5 mM $MgCl_2$) and 1U of *Taq* DNA polymerase (Bangalore Genei, India). DNA amplifications were performed in BioRad Dyad thermocycler with one cycle of initial denaturation (94°C, 1 min), annealing (35°C, 2 min) and extension (72°C, 2 min) with a final extension at 72°C for 5 min.

All the amplifications were repeated three times to check the reproducibility of the banding pattern. A negative control was included in each set of PCR reactions. PCR amplified products together with marker (1 Kb Fermentas, USA) were resolved by gel electrophoresis (4 Vcm^{-1}) on 1.4% agarose gels in 1X TAE buffer containing 0.5 mg mL^{-1} Et-Br and visualized under UV transilluminator.

IV. *Data analysis*

The fingerprints generated by different primers were compared for the relatedness among isolates. The presence (scored 1) or absence (scored 0) of a band of a particular molecular weight was scored as two alleles at single locus to compile binary matrices. The dendrogram were constructed by UPGMA cluster analysis using three different coefficients: the simple matching coefficient (SM), DICE coefficient and Jaccard coefficient (J). To provide another means of testing the relationships among isolates a three-dimensional common principal components analysis (CPCA) was constructed by using EIGEN module of NTSYS-pc (V2.02j, Applied Biostatistics Inc).

CHAPTER IV

RESULTS

I. Morphological identification and characterization of *M. phaseolina*

1. Isolation and identification

2. Pathogenecity and Pycnidium formation

3. Lyophilization and revival

II. Identification and detection of *M. phaseolina*

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3. Sequencing and sequence submission
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III. Molecular characterization and genomic fingerprinting

1. Genetic diversity obtained by 10-mer primers (Operon kit)

2. Genetic diversity obtained by BOXA1R primer

3. Genetic diversity obtained by M13 mini satellite primer

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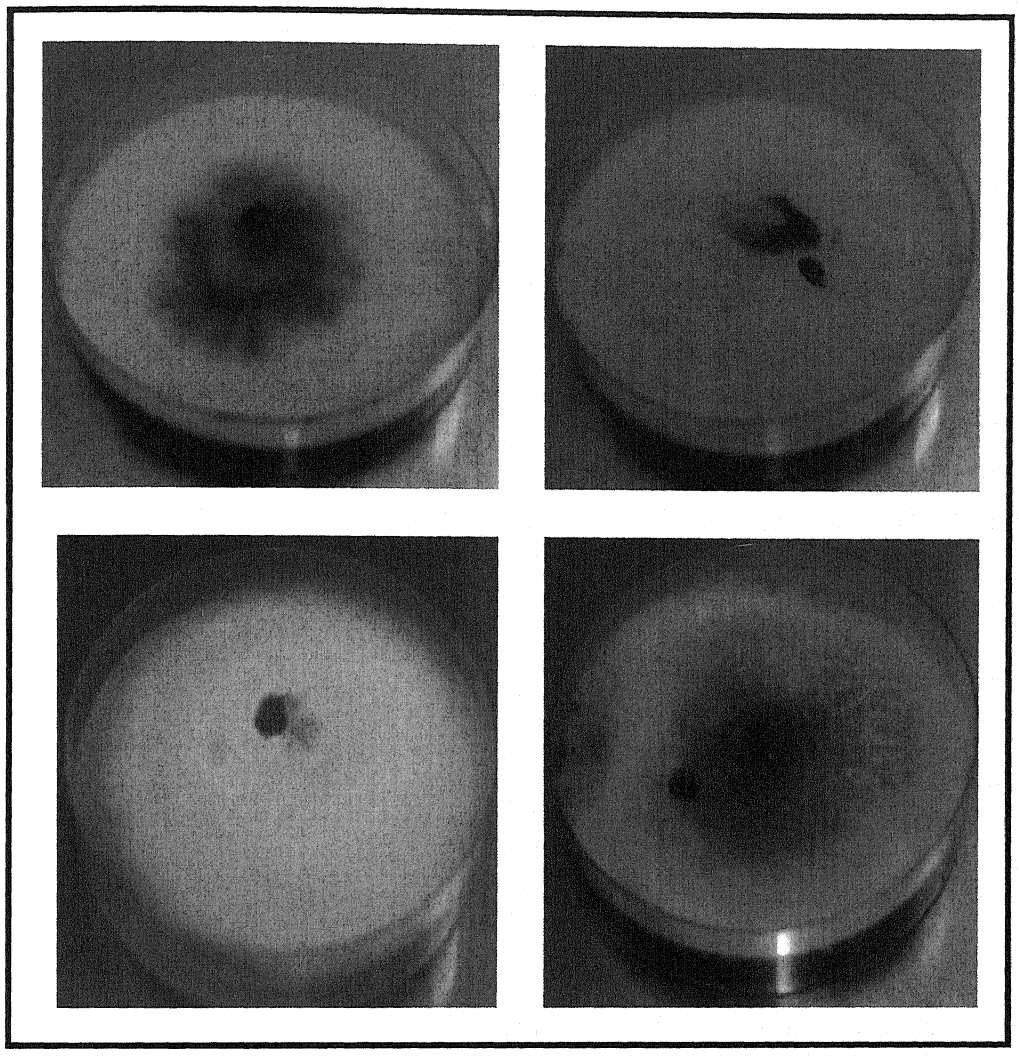
I. Morphological identification and characterization of *M. phaseolina*

Different isolates of *M. phaseolina* were isolated from root and stem of bean, chickpea, corn, soybean plants and also from the field soil (Table 3.1). In general, maximum number of isolates was obtained from chickpea plants. Some reference isolates of *M. phaseolina* isolates were obtained from UK-CABI culture collection. These isolates of *M. phaseolina* were characterized morphologically based on colony color and sclerotial formation (Fig. 4.1A and B).

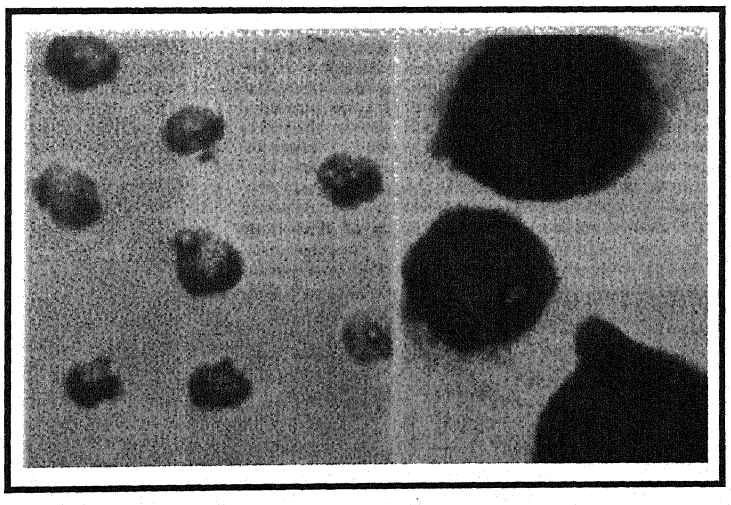
The colonies of *M. phaseolina* were generally identified by the grayish-brown to black in colour with abundant aerial mycelium and sclerotia intermingled amongst the hyphae. Some colonies appeared dark-brown to black having a characteristic granular appearance due to the presence of numerous sclerotia immersed under surface of the agar plates. Under microscope, the mycelium appeared both inter and intra-cellular. Pycnidia were produced from simple pycnidiogenous cells. Pycnidiogenous cells were enteroblastic-phialidic and were borne on septate-branched pycnidiophores.

I. 1. Pathogenecity and Pycnidium formation

All the 50 isolates were tested for pathogenecity under the controlled conditions. Nineteen isolates were pathogenic to more than one of their respective host plants; twenty-five were only pathogenic to one of their respective host plants, sorghum (mpk4, 6, 16, 20, 21, 31, 39, 41, 42and 44), chickpea (mpk3, 5, 11, 23, 33, 38, 40 and 48) and soybean (mpk7, 9, 24, 27, 34, 35 and 36), while six isolates were not pathogenic. However, none of the isolates showed any relation with pathogenecity in relation to their geographical origin.



A



B

Fig. 4.1 (A) Morphological similarity of *M. phaseolina* isolates (B) Pycinidia formation in *M. phaseolina*

The pathogenecity of isolates, obtained from the plant roots was compared with soil isolates and no difference was detected in their symptom expressions (Table 4.1).

Table 4.1 Pathogenecity and pycnidia formation by *M. phaseolina* isolates

Isolate No.	Host of origin	Pycnidia formation**	Pathogenecity *on different host		
			Sorghum	Chickpea	Soybean
mpk1	Potato	0	2	0	0
mpk2	Potato	2	0	0	0
mpk3	Chickpea	3	0	3	0
mpk4	Sorghum	0	3	0	0
mpk5	Chickpea	3	0	3	0
mpk6	Sorghum	0	3	0	0
mpk7	Soybean	3	0	0	3
mpk8	Corn	0	2	2	2
mpk9	Soybean	2	0	0	3
mpk10	Soil	2	0	0	0
mpk11	Chickpea	2	0	3	0
mpk12	Soil	0	2	2	2
mpk13	N.A	0	0	0	0
mpk14	Pea	0	0	0	0
mpk15	Corn	3	2	0	2
mpk16	Sorghum	2	3	0	0
mpk17	Chickpea	0	2	3	2
mpk18	Mung bean	2	0	2	2
mpk19	Soybean	3	2	2	3
mpk20	Sorghum	2	3	0	0
mpk21	Sorghum	0	3	0	0
mpk22	Corn	2	2	0	2
mpk23	Chickpea	3	0	3	0
mpk24	Soybean	2	0	0	3
mpk25	Pea	2	0	0	0
mpk26	Soil	0	2	0	2
mpk27	Soybean	2	0	0	3
mpk28		0	0	0	0
mpk29	N.A	0	2	2	2
mpk30	Ground nut	2	2	2	2
mpk31	Sorghum	3	3	0	0
mpk32	French bean	0	2	2	2
mpk33	Chickpea	3	0	3	0
mpk34	Soybean	2	0	0	3

mpk35	Soybean	0	0	0	3
mpk36	Soil	0	0	0	3
mpk37	Soybean	2	2	2	2
mpk38	Chickpea	3	0	3	0
mpk39	Sorghum	2	3	0	0
mpk40	Chickpea	3	0	3	0
mpk41	Sorghum	2	3	0	0
mpk42	Sorghum	2	3	0	0
mpk43	Corn	3	0	2	2
mpk44	Sorghum	2	3	0	0
mpk45	Sorghum	2	2	2	2
mpk46	Corn	2	0	2	2
mpk47	Soybean	0	3	3	3
mpk48	Chickpea	3	0	3	0
mpk49	Sunflower	2	2	0	2
mpk50	Corn	0	2	2	2

Note: * Pathogenecity of 50 isolates was tested on the hosts (sorghum, chickpea and soybean). ** Pycnidium formation tested on leaf tissues of the same host of origin. Pathogenecity scores on scale 3 to 0 indicates frequent, infrequent and non-pathogenic, similarly, pycnidia formation scores on scale 3 to 0.

Out of fifty isolates, pycnidium production did not occurred in 17, while 33 produced pycnidium in their host plants. In chickpea isolates, pycnidium formation was significantly greater than corn, bean and soybean. Most of the bean isolates formed pycnidia on leaf tissues; however, they never matured to produce pycnidiospore even with extended incubation (Table 4. 1).

I. 3. Lyophilization and revival

All lyophilized *M. phaseolina* cultures were revived and isolates were submitted to the repository of National Bureau of Agriculturally Important Microorganisms (NBAIM), India (Table 3.1).

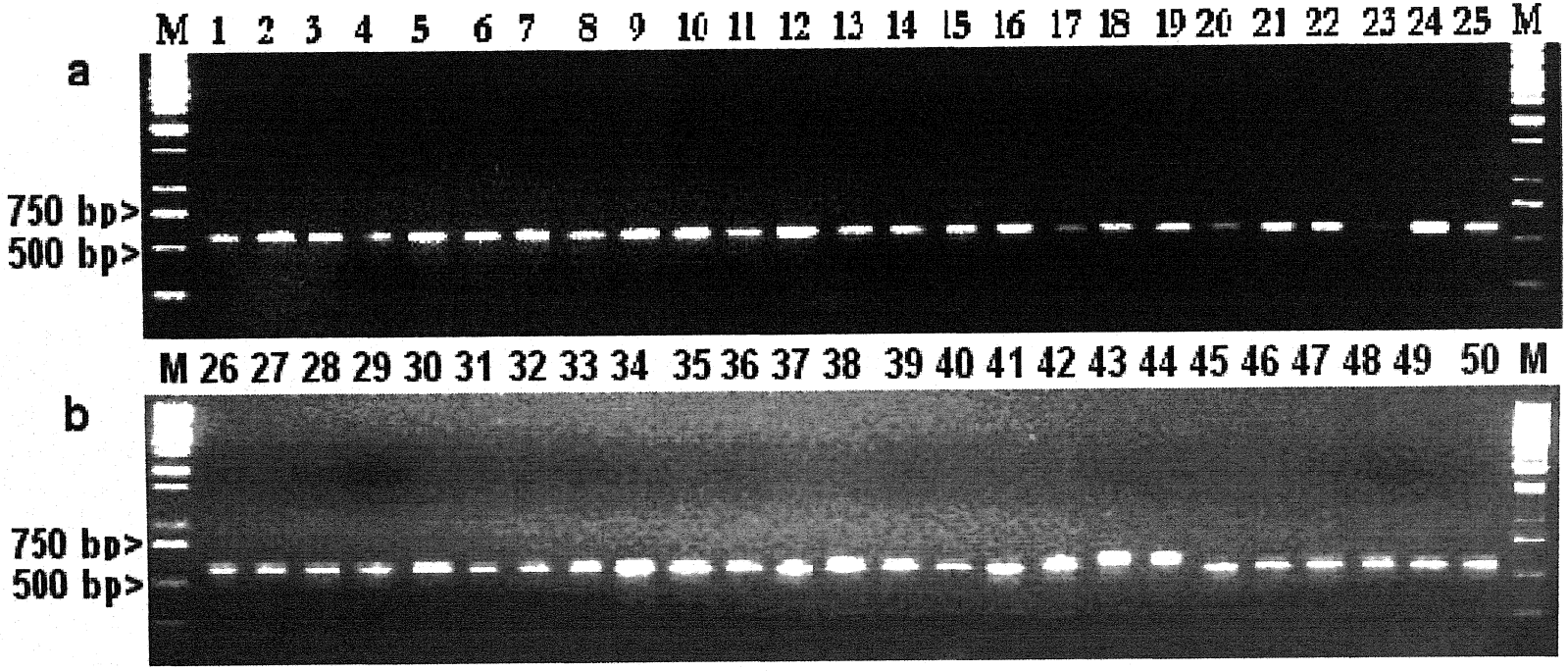


Fig. 4.2 PCR Amplification of rDNA gene cluster: Primers ITS-1 and ITS-4 were used for amplification of nearly 650 bp fragment of rDNA gene cluster, consisting of ITS-1, the 5.8S rDNA gene and ITS-2 region. (a) Lane1 to 25 and, (b) Lane 26 to 50 are the amplified products of *M. phaseolina* isolates (Table 3.1). M- 1kb molecular ladder.

II. Identification and detection of *M. phaseolina*

The PCR-based identification and detection of different isolates of *M. phaseolina* was done by the development of species-specific oligonucleotide primers and probe by exploiting the internal transcribed spacer (ITS) and the genetic diversity was estimated by using RAPD-PCR technique. Thirteen RAPD 10-mer primers, (OPA-15, OPA-11, OPA-10, OPB-08, OPV-17, OPC-10, OPB-07, OPB-06, OPD-11, OPB-17, OPE-01, Oligo-09 and OPA-09) (Table 3.5), two URP primers (URP-9F and URP-P1F), one rep-primer (BOXA1R) and one M13 mini-satellite primer were analyzed (Table 3.2). The PCR coupled to restriction fragment length polymorphism (RFLP) was also used.

II. 1. Internal Transcribed Spacer Region

5.8S rDNA gene amplification

Genomic DNA amplification by using ITS1 and ITS4 primers yielded a fragment approximately 650 bp that consists of ITS-1, 5.8S and ITS-2 region of rDNA gene cluster of (Fig. 4.2). The variability within the ITS amplified regions was also investigated by cleaving this fragment with 20 different restriction enzymes (Table 3.4). The restriction enzymes *Eco*R I, *Sau* 3A I, *Alu* I and *Cla* I produced identical pattern for all the isolates. The enzymes *Hap* II, *Msp* I and *Taq* I have multiple cleavage sites in ITS amplified product and produced fragments less than 100 bp in size. The enzymes *Apa* I, *Hind* III and *Sac* I failed to cleave the ITS region (Fig. 4.3).

II. 2. 28S rDNA gene cluster amplification

To study the polymorphism at 28S rDNA gene region a set of primers ITS1 and P3 were used in PCR amplification. Around 1300 bp fragment was obtained and was subjected for restriction digestion analysis by using *Hpa* I restriction enzyme, which yielded a fragment size of 600 bp, 400 bp and 300 bp in all the

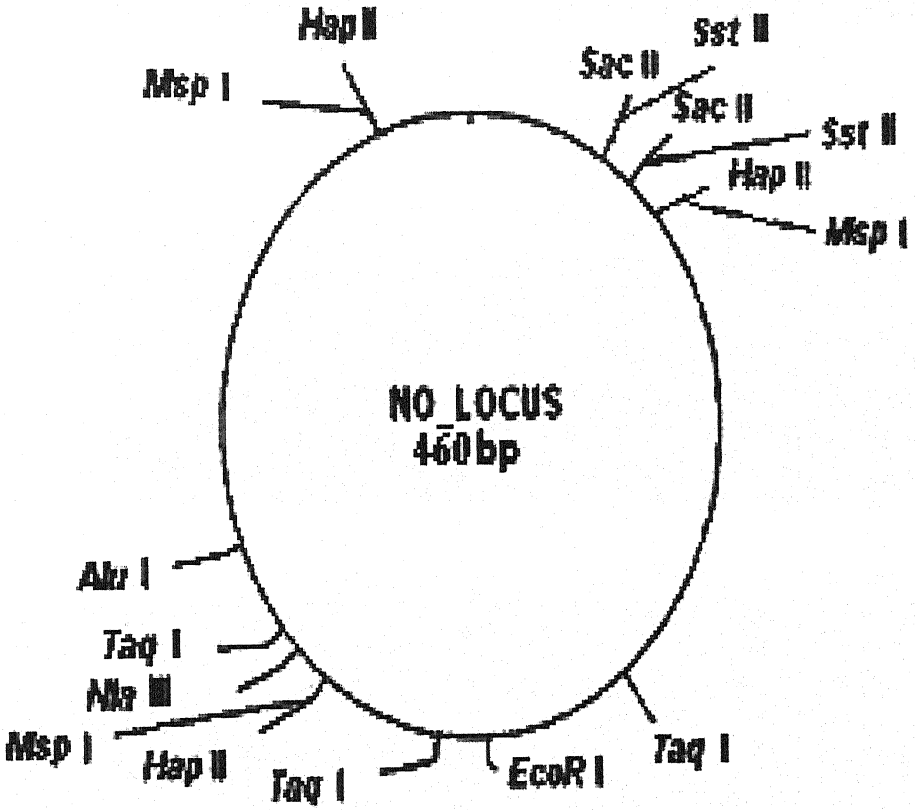
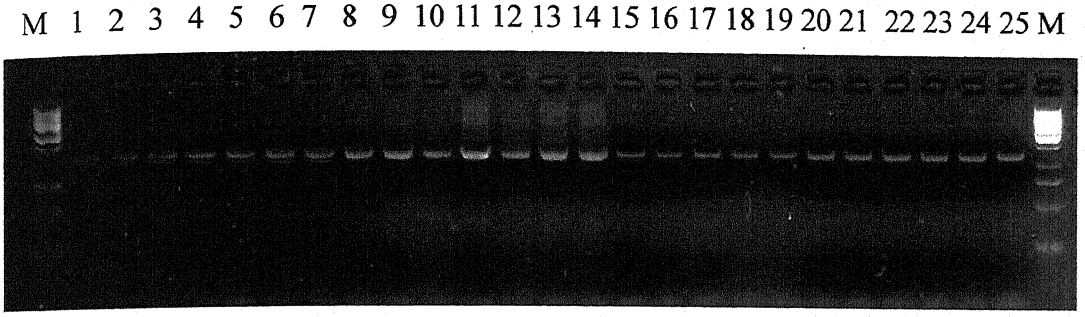
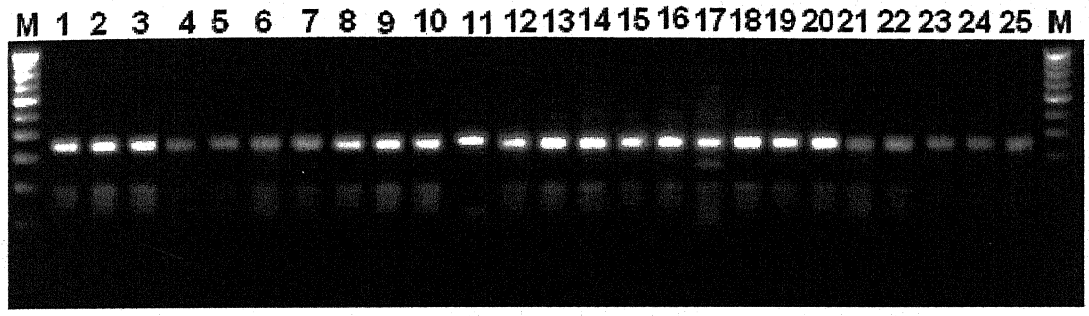
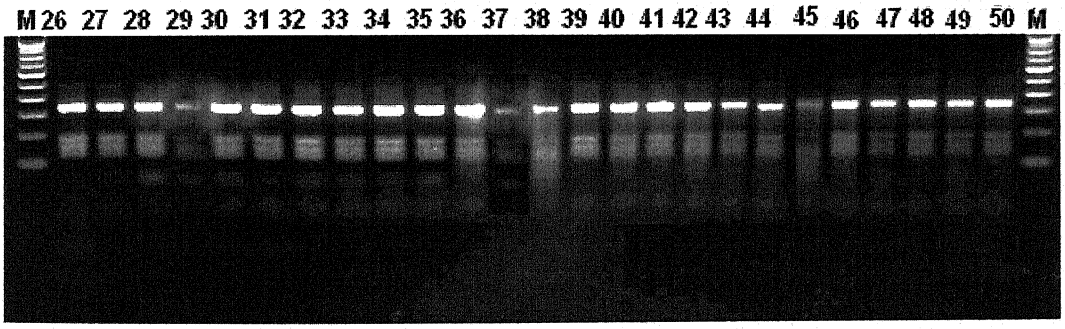


Fig. 4.3 Restriction map showing different restriction enzyme sites within the 5.8 sRNA gene, ITS-1 and ITS-2, the total length of the fragment is 460 bp.



A





B

Fig. 4.4 (A) PCR-Amplification of 28S rDNA gene.
(B) Restriction fragment length polymorphism using *Hpa*I.

isolates of *M. phaseolina* (Fig. 4.4a and b) Therefore, digestion of amplified ITS region with different restriction enzymes did not produce significant polymorphic pattern among the isolates.

II. 3. Sequencing and sequence submission

The sequences of ITS region for eight isolates was analyzed for homologies and was deposited in the GenBank database (Table 4.2)

Table 4.2 List of *M. phaseolina* cultures used for sequencing

Sl. No	Biological origin Year of collection	Geographical origin	NBAIM Acce.No.	GenBank Acces.No.
1	Chickpea-2001	Karnataka, India.	F1281	DQ359737
2	Sorghum-2003	Andhra Pradesh, India	F1275	DQ359738
3	Soybean -2003	Karnataka, India	F1273	DQ359739
4	Soil -2003	Varanasi, Uttar Pradesh, India	F1262	DQ359740
5	Potato-2000	Himachal Pradesh, India	F1289	DQ359741
6	Sorghum-2000	Delhi (IARI), India	F1291	DQ359742
7	*Phaseolus mungo*-2003	Delhi (IARI), India	F1267	DQ359743
8	Chickpea-2002	Varanasi, Uttar Pradesh, India	F1263	DQ359744

The FASTA format of ITS sequences of *M. phaseolina*

>gi|85838223|gb|DQ359737.1| *Macrophomina phaseolina* isolate KB-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

CCACCCTTTGTATACCTACCTCTGTTGCTTTGGCGTGCCGCGGTCTTCCGCGGCCGCC
CCCCGGATTTGGGGGGTGGCTAGTGCCCGCCAGAGGACTATCAAACTCCAGTCAGTA
AACGTTGCAGTCTGAAAAAAATATTAAATAAACTAAAACTTTCAACAACGGATCTCT
TGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGG
GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCACCGTC
CTTTGCGGGCGCGCCTCAAAGACCTCGGCGGTGGCGTCTTGCCTCAAGCGTAGTAGA
ATACACCTCGCTTCGGAGCGTAAGGCGTCGCCCGCCGGACGAACCTTCTGAACTTTT
CTCAAG

>gi|85838224|gb|DQ359738.1| *Macrophomina phaseolina* isolate KB-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

TCCCACCCTTTGTATACCTACCTCTGTTGCTTTGGCGGGCCGCGGTCTTCCGCGGCCG
CCCCCCGATTTTGGGGGGTGGCTAGTGCCCGCCAGAGGACTATCAAACTCCAGTCAG
TAAACGTTGCAGTCTGAAAAAAATATTAAATAAACTAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGG
GGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCACCGT
CCTTTGCGGGCGCGCCTCAAAGACCTCGGCGGTGGCGTCTTGCCTCAAGCGTAGTAG
AATACACCTCGCTTCGGAGCGTAAGGCGTCGCCCGCCGGACGAACCTTCTGAACTTT
TCTCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA
AGGGGAGGAA

>gi|85838225|gb|DQ359739.1| *Macrophomina phaseolina* isolate KB-3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

TTTGTATACCTACCTCTGTTGCTTTGGCGGGCCGCGGTCTTCCGCGGCCGCCCCCCGA
TTTTGGGGGGTGGCTAGTGCCCGCCAGAGGACTATCAAACTCCAGTCAGTAAACGTT
GCAGTCTGAAAAAAATATTAAATAAACTAAAACTTTCAACAACGGATCTCTTGGTTC
TGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGC
CTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCACCGTCCTTTGC
GGGCGCGCCTCAAAGACCTCGGCGGTGGCGTCTTGCCTCAAGCGTAGTAGAATACAC
CTCGCTTCGGAGCGTAAGGCGTCGCCCGCCGGACGAACCTTCTGAACTTTTCTCAAG
GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGGGGAG
GAA

>gi|85838226|gb|DQ359740.1| *Macrophomina phaseolina* isolate KB-4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

GAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATT
CATGGCCGCCGGGGGCTCTCAGCCCCGGGATTTTGGGGGGTGGCTAGTGCCCGCCAG
AGGACTATCAAACTCCAGTCAGAAACGTTGCAGTGCGAAAAAAATATTAAATAAAC
TAAAACTTTCAACAATGGATCTTTTGGTTANGGCATCAGATGAAGAACGCAGCGAAA
TGCAATAAGTAATNTGAATTGCTGAATTCATTGAATCATGGAATCTTTGAACGCACA
TTGNGCCCCGTGCTATTCCGGGGGGCATGCCTGTTCTGAGCGTCATTTCAACCCTCGA
GCTCTGGTTGTTATTGGGCTACCGTCCTGTGCGGGCGCCGCCATCAAAGAGCCTGCG
GGCGGTGGCGTCTTGCCTCAAGGGTAGTAGAATACACCTCGCTTCGGAGCAGTACGG
CGTCGCCCGCNGGAGAGAACCATTTTA

>gi|85838227|gb|DQ359741.1| *Macrophomina phaseolina* isolate KB-5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

GCCGCGGTCTTCCGCGGCCGCCCCCCGATTTTGGGGGGTGGCTAGTGCCCGCCAGAG
GACTATCAAACTCCAGTCAGTAAACGTTGCAGTCTGAAAAAAATATTAAATAAACTA
AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTC
TGCTTGGTATTGGGCACCGTCCTTTGCGGGCGCGCCTCAAAGACCTCGGCGGTGGCG
TCTTGCCTCAAGCGTAGTAGAATACACCTCGCTTCGGAGCGTAAGGCGTCGCCCGCC
GGACGAACCTTCTGAACTTTTCTCAAGG

>gi|85838228|gb|DQ359742.1| *Macrophomina phaseolina* isolate KB-6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

TCCCACCCGTTTGTATACCTACCTCTGTTGCTTTGGCGGGCCGCGGTCTTCCGCGGCC
GCCCCCCGATTTTGGGGGGTGGCTAGTGCCCGCCAGAGGACTATCAAACTCCAGTCA
GTAAACGTTGCAGTCTGAAAAAAATATTAAATAAACTAAAACTTTCAACAACGGATC
TCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGG
GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCACCG
TCCTTTGCGGGCGCGCCTCAAAGACCTCGGCGGTGGCGTCTTGCCTCAAGCGTAGTA
GAATACACCTCGCTTCGGAGCGTAAGGCGTCGCCCGCCGGACGAACCTTCTGAACTT
TTCTCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAT
AAGGGAAGGAA

>gi|85838229|gb|DQ359743.1| *Macrophomina phaseolina* isolate KB-7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

TCCCACCCTTTGTATACCTACCTCTGTTGCTTTGGCGGGCCGCGGTCTTCCGCGGCCG
CCCCCCGATTTTGGGGGGTGGCTAGTGCCCGCCAGAGGACTATCAAACTCCAGTCAG
TAAACGTTGCAGTCTGAAAAAAATATTAAATAAACTAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGG
GGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCACCGT
CCTTTGCGGGCGCGCCTCAAAGACCTCGGCGGTGGCGTCTTGCCTCAAGCGTAGTAG
AATACACCTCGCTTCGGAGCGTAAGGCGTCGCCCGCCGGACGAACCTTCTGAACTTT
TCTCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA
AGGGGAGGAA

>gi|85838230|gb|DQ359744.1| *Macrophomina phaseolina* isolate KB-8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

TCCCACCCTTTGTATACCTACCTCTGTTGCTTTGGCGGGCCGCGGTCTTCCGCGGCCG
CCCCCCGATTTTGGGGGGTGGCTAGTGCCCGCCAGAGGACTATCAAACTCCAGTCAG
TAAACGTTGCAGTCTGAAAAAAATATTAAATAAACTAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGG

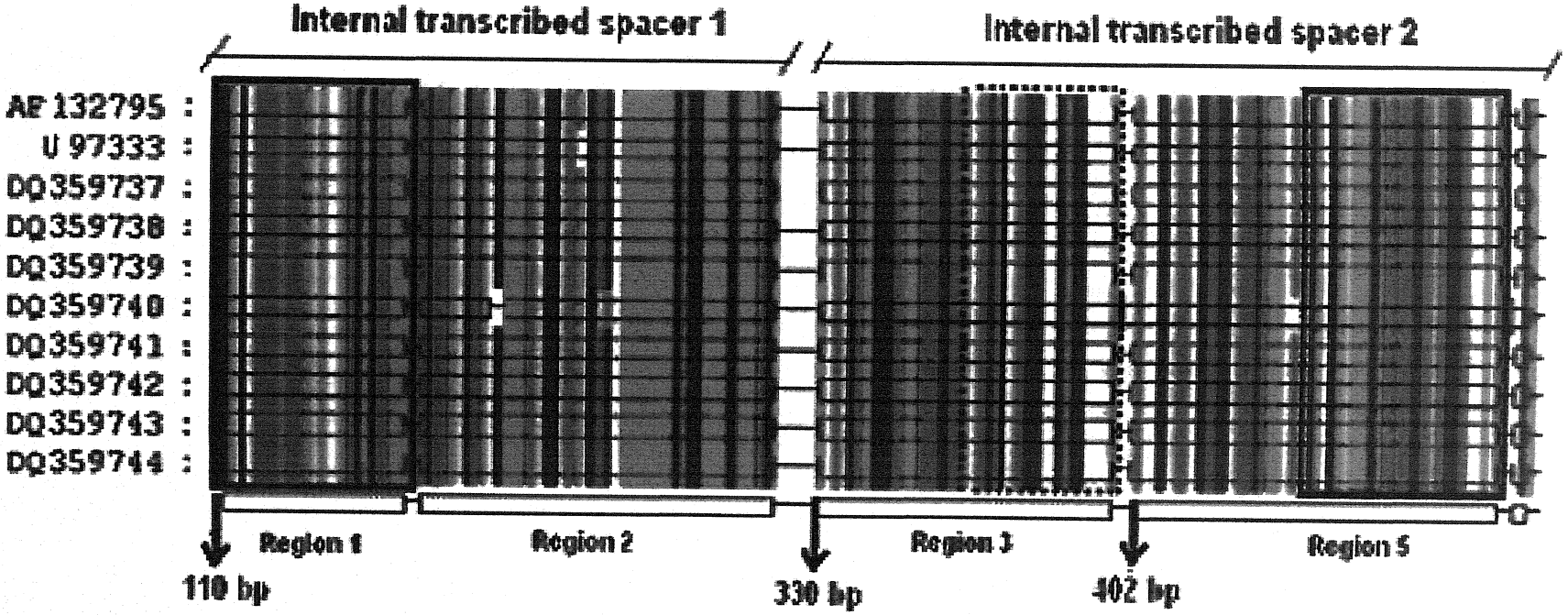


Fig. 4.5 Development of specific oligonucleotide primers and probe: Alignment of ITS-1 and ITS-2 sequences from eight isolates of *Macrophomina phaseolina* and two reference sequences (AF132795 and U97333) taken from GenBank database. Nucleotides are shown in color bars (A-red, G-yellow, T-blue and C-green). The regions 1, 2, 3 and 5 are completely aligned, 5.8 S RNA gene is not shown and because of variability the region 4 was omitted. The solid line rectangles indicate specific nucleotide areas used for the development of specific oligonucleotide primers and the dashed rectangles shows the specific region used for designing of the probe. The position of the first nucleotide of region 1 and others were given according to the reference sequence AF132795 (Table 4.2).

GGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCACCGT
CCTTTGCGGGCGCGCCTCAAAGACCTCGGCGGTGGCGTCTTGCCTCAAGCGTAGTAG
AATACACCTCGCTTCGGAGCGTAAGGCGTCGCCCGCCGGACGAACCTTCTGAACTTT
TCTCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA
AGGGGAGGAA

II. 4. Designing and testing of oligonucleotide primers and probe

Sequence comparison of ITS region encompassing ITS-1, 5.8S rRNA gene and ITS-2 of *M. phaseolina* and other related fungal species revealed three regions that were conserved among the *M. phaseolina* isolates. The complete sequence was virtually divided into five regions from ITS-1 to ITS-2 (Fig. 4. 5). The region 4 that showed variability among the aligned sequences of *M. phaseolina* isolates was not considered for further analysis. The 5.8S RNA gene sequence was also excluded from the analysis.

Table 4.3 Species specific oligonucliotides designed

Sl. No	Primers	Sequence
1	MPKF1	5'-CCG CCA GAG GAC TAT CAA AC-3'
2	MPKR1	5'-CGT CCG AAG CGA GGT GTA TT-3'
3	MPKH1	5'-GCT CTG CTT GGT ATT GGG C-3'

After editing and rearrangement of aligned sequences and comparison with the sequences of closely related genera of fungi, region 1 and 5 were selected for the development of species-specific primers for *M. phaseolina.* Two primers MpKFI and MpKRI were designed from the specific nucleotide areas and one oligonucleotide probe MpKH1 (Table 4.3) was designed from the conserved region, adjacent to 5.8S gene showed in the region 3 (Fig. 4. 5).

The designed primers yielded single amplified product of 350 bp with all the *M. phaseolina* isolates. The specificity of the primers was tested on representative species of common soil-borne fungi, bacteria and actinomycetes (Table 4.4). The

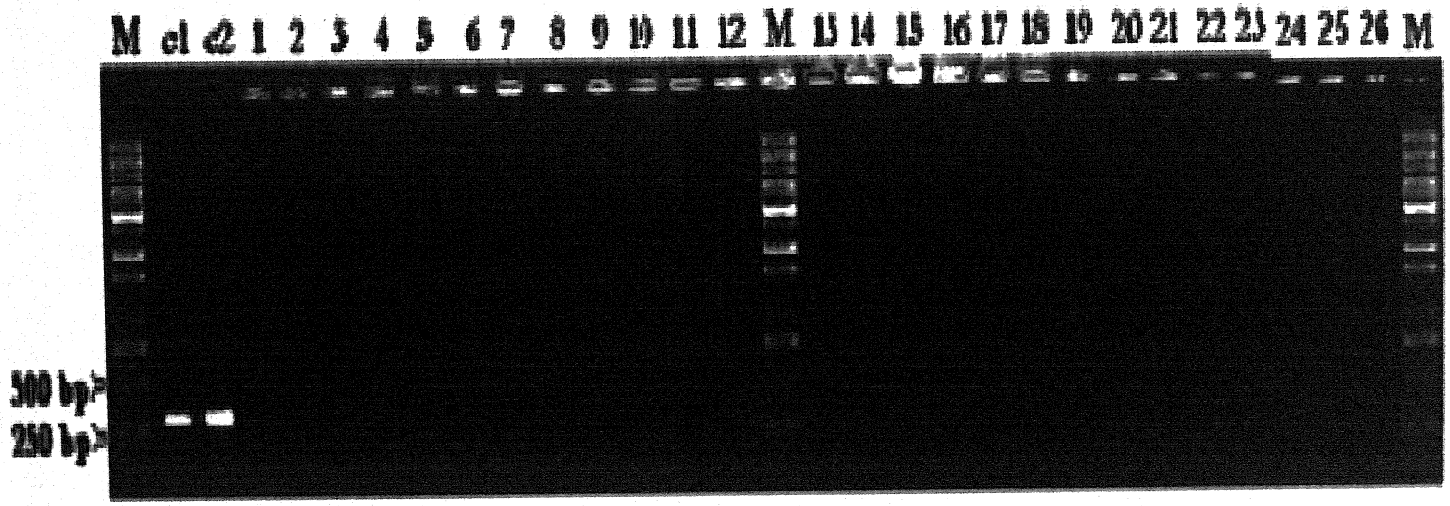


Fig. 4.6 Testing of primers MpKF1 and MpKR1: PCR amplification of *M. phaseolina* produced 350bp amplicon lane c1and c2. Lane 1 to 26 showing no amplified product with different test microbes (Table 4.4). M represents 1 Kb ladder.

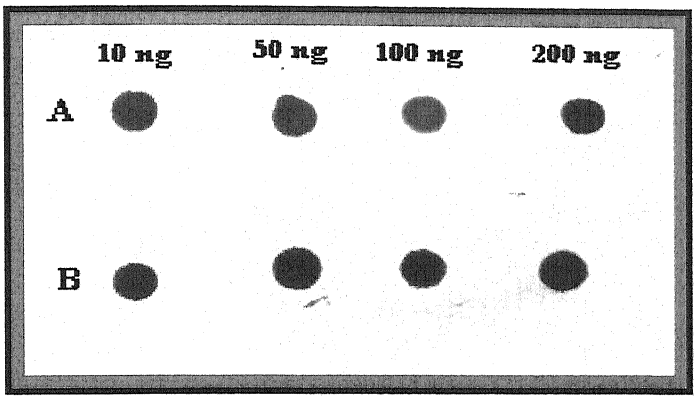
primer pair was found to be specific for *M. phaseolina* as none of the other microbes yielded amplified products under the identical conditions of amplification as described earlier (Fig. 4. 6).

Table 4.4 Different groups of microorganisms used for the testing of specific oligonucleotide primers and probe.

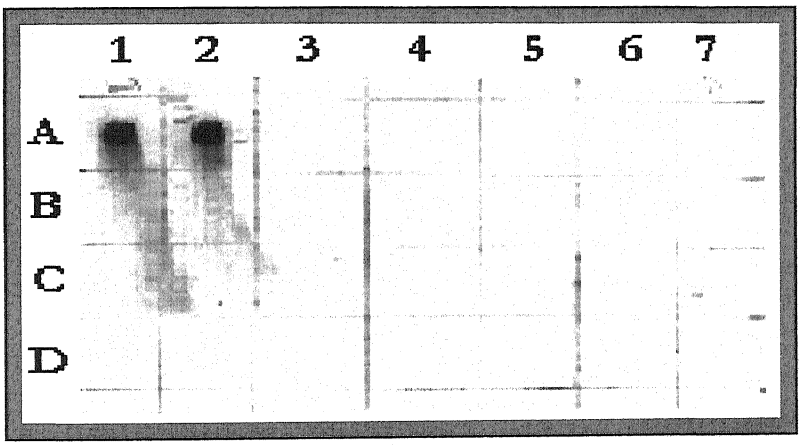
Sl. No.	Microbial culture	NBAIM Acc. No.
1	*Fusarium oxysporum*	F486
2	*Fusarium udum*	F919
3	*Fusarium moliniformae*	F291
4	*Neurospora crassa*	F1425
5	*Alternaria alternata*	F143
6	*Alternaria brassicicola*	F076
7	*Trichoderma viride*	F1316
8	*Aspergillus niger*	F583
9	*Aspergillus oryzea*	F374
10	*Chaetomium globosum*	F444
11	*Trametes lactinea*	F1852
12	*Pencillium brevicompactum*	F1496
13	*Verticillium lecanii*	F2102
14	*Phytophthora cambivora*	F1648
15	*Rhizopus oryzae*	F1747
16	*Beauveria bassiana*	F298
17	*Sclerotium rolfsii*	F1766
18	*Rhizoctonia solani*	F1723
19	*Metarhizium anisopliae*	F1311
20	*Phialophora calciformis*	1093*
21	*Psuedomonas putida*	B231
22	*Psuedomonas fluorescence*	B223
23	*Bacillus subtilis*	B082
24	*Arthrobacter citreus*	
25	*Arthrobacter* spp.	
26	*Streptomyces* spp.	

*NCIM-National Collection of Industrial Microorganisms. India.

Detectable hybridization signal was obtained with an oligonucleotide probe at 1 pmol mL^{-1} concentration; pre-hybridization, hybridization and washing steps



a



b

Fig. 4.7 Dot blot hybridization: a). Hybridization of oligonucleotide probe MpKH1 with different concentrations of rDNA amplified product from two different strains of *M. phaseolina.* **b).** Hybridization of oligonucleotide probe MpKH1 with rDNA amplified products from representative strains of different microbial groups. Lane representing A1 and A2 are positive controls, A3 to D7 are test microbes listed in the Table 4.4.

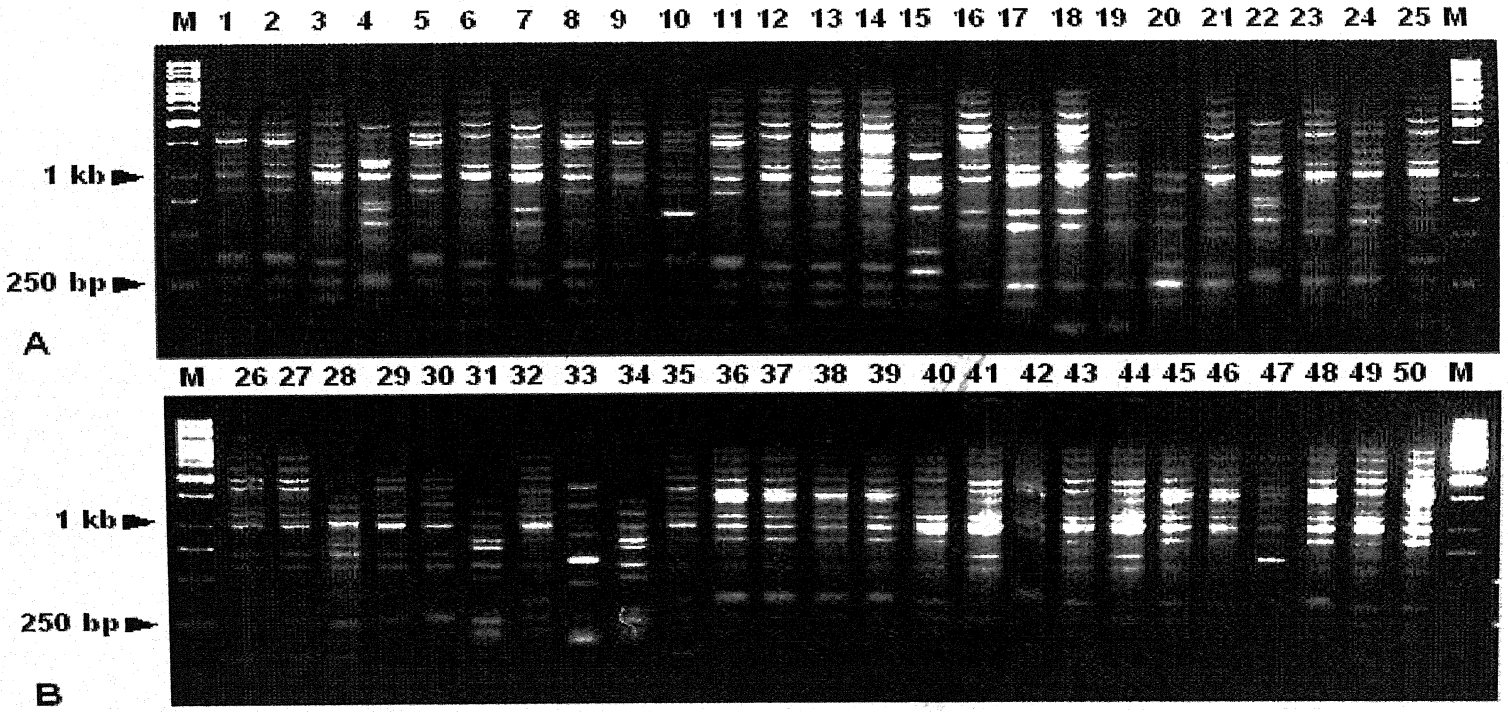


Fig. 4.9 RAPD-PCR fingerprinting: 50 isolates of *M. phaseolina* collected from different geographical regions of India were subjected to PCR amplification by using 10-mer RAPD primer OPB-8. (A) Lane 1 to 25 and (B) Lane 26 to 50 indicating isolates of *M. phaseolina* (Table 3.1). M-Represents1Kb ladder. ◄- Indicates monomorphic bands present in all isolates.

Table 4.5 Clustering of isolates based on SSCP banding pattern

Sl.No	SSCP banding type	List of isolates
1	Type I	mpk2, mpk3, mpk5, mpk7, mpk8, mpk9, mpk10, mpk11, mpk12, mpk13, mpk14, mpk15, mpk16, mpk17, mpk18, mpk19, mpk20, mpk21, mpk22, mpk22, mpk23, mpk24, mpk25, mpk26, mpk27, mpk28, mpk29, mpk30, mpk31, mpk32, mpk33, mpk34, mpk35, mpk36 and mpk37.
2	Type II	mpk1 and mpk4.
3	Type III	mpk6, mpk38, mpk39, mpk40, mpk41, mpk42, mpk43, mpk44, mpk45, mpk46, mpk47, mpk48, mpk49 and mpk50.

III. Molecular characterization and genomic fingerprinting by RAPD-PCR

In order to study the genetic relationships between the 50 isolates and to obtain a robust dendrogram, we used thirteen 10-mer arbitrary primers from Operon kit and three other random primers. Name and sequence of primers, total number of amplified bands and number of polymorphic bands and percentage of polymorphic bands of each primer was mentioned in the table 4.6. The dendrogram obtained using SM, DICE and J coefficients showed the same structure and the same cluster. Therefore, we have only presented the results obtained with the J coefficient.

Each of the thirteen RAPD 10-mer primers produced banding patterns ranging from 250 bp to 3.5 Kb. Primers which were showing no significant correlation and produced similar type of results was listed in the table 4.6, and was not discussed further.

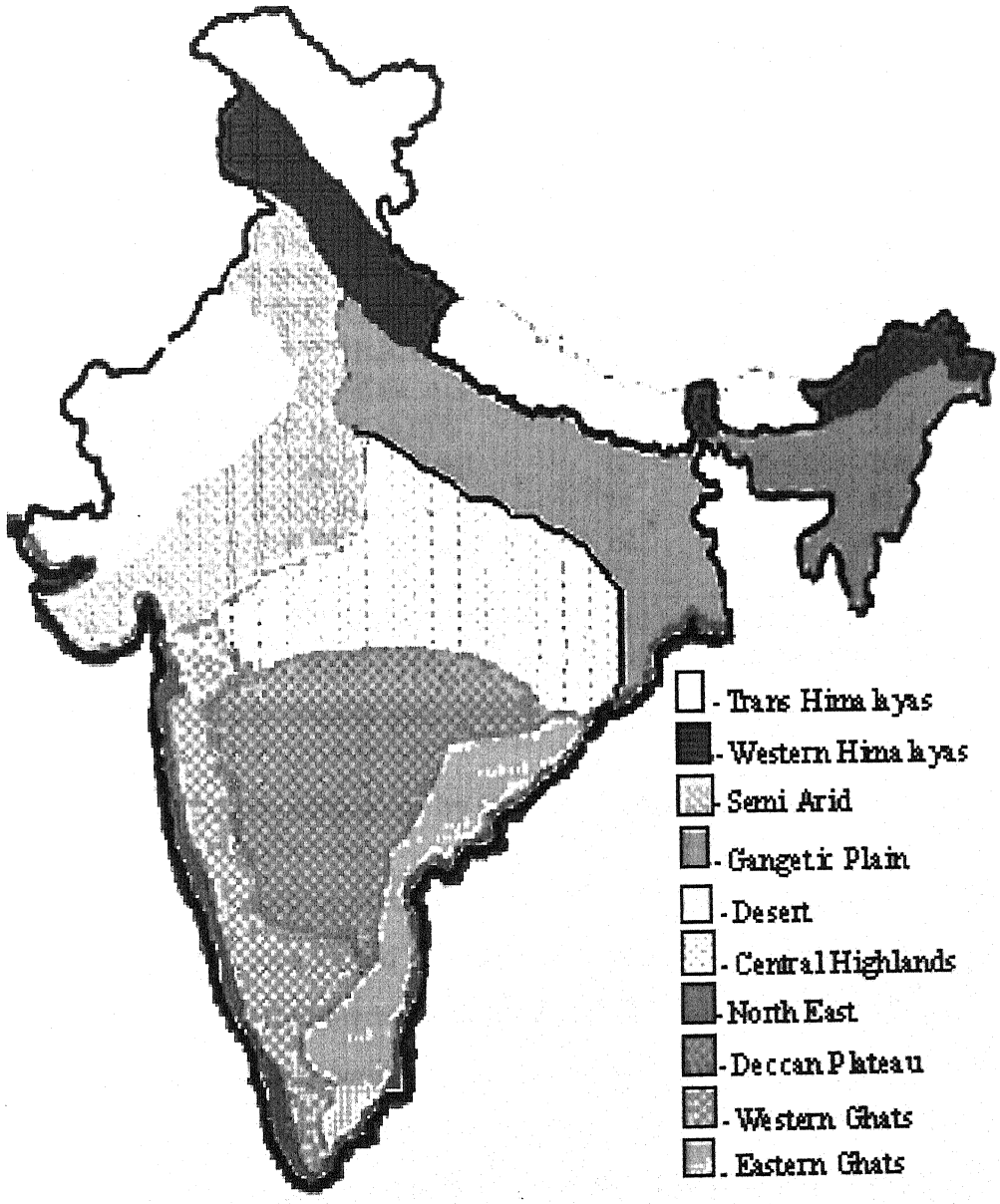


Fig. 4.10 Map of India showing major Agro-Climatic zones. Details of *M. phaseolina* isolates were given in the Table. 4.7. Samples were not collected from the regions Trans Himalayas and Desert.

Table 4.6 List of 10-mer random primers obtained from Operon kit

Primer	Sequence (5′ to 3′)	TNB	NPB	P%
OPA-15	TTCCGAACCC	08	5	62.5
OPA-11	CAATCGCCGT	15	12	80
OPB-08	GTGAGCTAGG	18	15	83.4
OPV-17	GACCGCTTGT	12	10	83.3
OPA-10	GTGATCGCAG	4	4	100
OPC-10	TGTCTGGGTG	06	4	66.7
OPB-06	TGCTCTGCCC	10	4	40
OPB-07	GGTGACGCAG	14	12	85.7
OPD-11	AGCGCCATTG	07	5	71.4
OPB-17	AGGGAACGAG	12	12	100
OPA-09	GGGTAACGCC	11	9	81.8
OPE-01	ACCGCGAAGG	12	10	83.3
Oligo-09	GTGATCGCA	17	15	

The total number of bands (TNB), number of polymorphic bands (NPB) and percentage of polymorphic bands (P %) obtained with each primer.

III. 1. Genetic diversity obtained by OPB-08 primer

Essentially the banding pattern produced by the OPB-8 ranged between 100 bp to 3 kb (Fig. 4.9 A and B) and the UPGMA clustering produced a dendrogram that separated the 50 isolates in to 10 groups at 30% similarity level (Fig. 4.11). The ten clusters correlated well with the geographical locations with exceptions for isolates obtained from Eastern and Western Ghats. All the isolates based on cluster analysis were listed in table (4.7). There was a segregation of isolates from these two geographical locations in to two clusters thus, distributing 10 genotypes in to eight geographical locations in India (Fig. 4.10). In each group, isolates shared 50 to 100% similarity among themselves. With in each geographical cluster (G.C), however no correlation was obtained with regards to biological origin, for example isolates obtained from sorghum (mpk39), chickpea (mpk3) and corn (mpk 8) from Indo-Gangetic Plains (G.C II) showed 100%

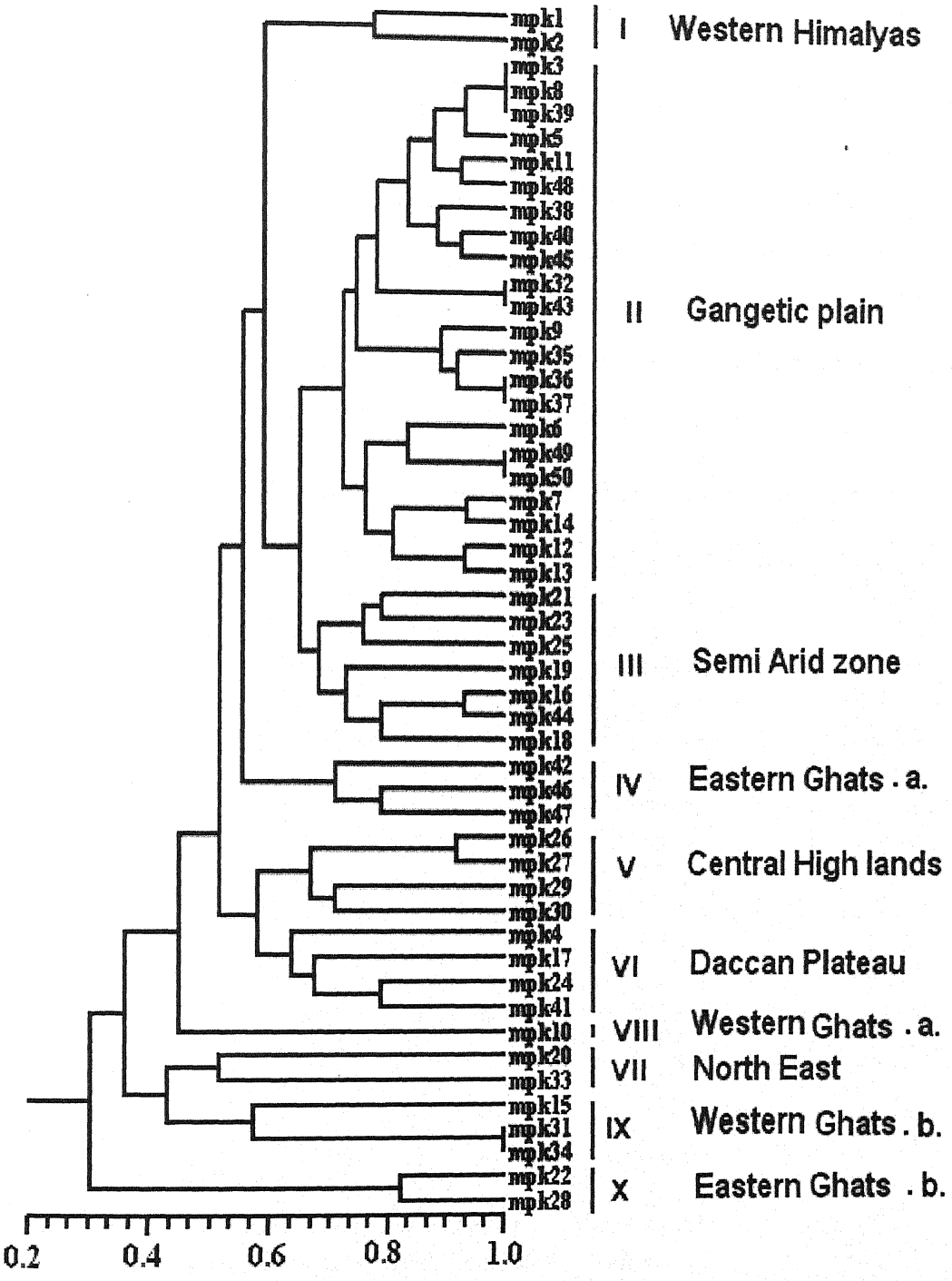


Fig. 4.11 UPGMA-SAHN clustering dendrogram: Constructed by the data obtained from the primer OPB-8 in RAPD assay of 50 *M. phaseolina* isolates labeled as mpk1 to mpk 50. Geographical clusters I to X genetic sub groups are representing ten geographic clusters. Scale in the dendrogram shows the genetic similarity coefficient

similarity among themselves. Similarly, all isolates from Semi arid zone (G.C III) produced similar banding patterns even though they were from sorghum, chickpea, pea, soybean and mung bean. Conversely, the isolates obtained from the same host like chickpea from Indo-Gangetic Plains did not showed 100% similarity among themselves. All the isolates of *M. phaseolina* irrespective of their host and geographical origin, exhibited two representative monomorphic bands at 250 bp and 1 kb (Fig. 4. 9 A and B).

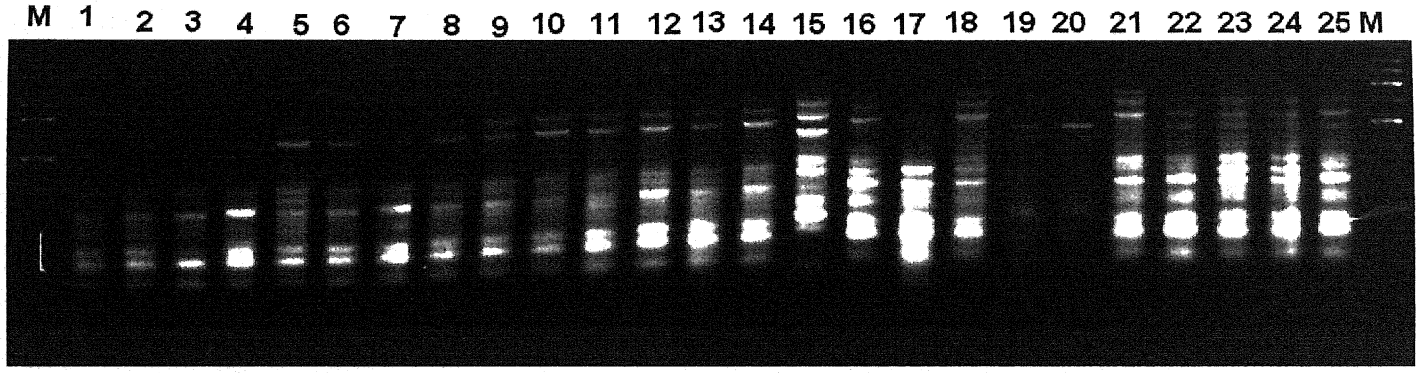
Table 4.7 Geographical region wise list of *M. phaseolina* isolates

Name	Geographical origin	Biological origin	Code no
Gangetic plain	Varanasi, Uttar Pradesh	Sorghum	mpk45
	Varanasi, Uttar Pradesh	Sorghum	mpk6
	Ballia, Uttar Pradesh	Sorghum	mpk39
	Varanasi, Uttar Pradesh	Chickpea	mpk 5
	Varanasi, Uttar Pradesh	Chickpea	mpk11
	Varanasi, Uttar Pradesh	Chickpea	mpk48
	Varanasi, Uttar Pradesh	Chickpea	mpk38
	Mau, Uttar Pradesh	Chickpea	mpk40
	Ballia, Uttar Pradesh	Chickpea	mpk3
	Varanasi, Uttar Pradesh	French bean	mpk32
	Uttar Pradesh	Corn	mpk43
	Ballia, Uttar Pradesh	Soybean	mpk9
	Varanasi, Uttar Pradesh	Soybean	mpk35
	Jhansi, Uttar Pradesh	Soil	mpk36
	Uttar Pradesh	Soybean	mpk37
	Mau, Uttar Pradesh	Corn	mpk8
	Varanasi, Uttar Pradesh	Sunflower	mpk49
	Jhansi, Uttar Pradesh	Corn	mpk50
	Jhansi, Uttar Pradesh	Soybean	mpk7
	Varanasi, Uttar Pradesh	Pea	mpk14
	Mau, Uttar Pradesh	Soil	mpk12
	Uttar Pradesh	N.A	mpk13
Semi Arid	Delhi	Sorghum	mpk21
	Delhi	Chickpea	mpk23
	Delhi	Pea	mpk25
	Delhi	Soybean	mpk19
	Gujarat	Sorghum	mpk16

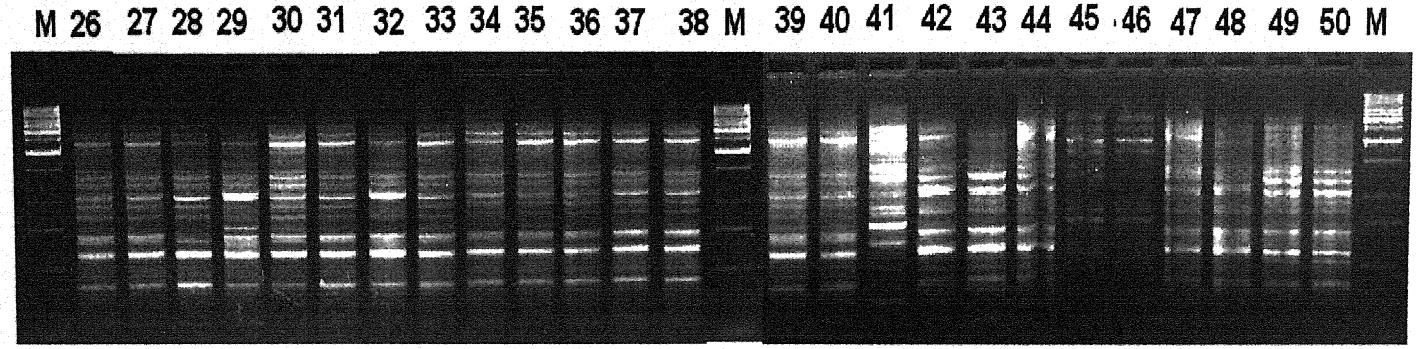
	Rajasthan	Sorghum	mpk44
	Delhi	Mung bean	mpk18
Central High lands	Madhya Pradesh	Soil	mpk26
	Madhya Pradesh	Soybean	mpk27
		N.A	mpk29
	Madhya Pradesh	Ground nut	mpk30
Daccan Plateau	Maharashtra	Sorghum	mpk4
	Karnataka	Chickpea	mpk17
	Andhra pradesh	Soybean	mpk24
	Gulberga, Karnataka	Sorghum	mpk41
NorthEast	Jorhat, Assam	Sorghum	mpk20
	Jorhat, Assam	Chickpea	mpk33
Eastern Ghats	Andhra Pradesh	Sorghum	mpk42
a	Andhra Pradesh	Corn	mpk46
	Chenni	Soybean	mpk47
b	Chennai	Corn	mpk22
	Tamil Nadu	N.A	mpk28
Western Ghats	Karnataka	Corn	mpk15
a	Solapur, Maharashtra	Sorghum	mpk31
	Karnataka	Soybean	mpk34
b	Kerala	Soil	mpk10
Western Himalayas	Himachal Pradesh	Potato	mpk1
	Himachal Pradesh	Potato	mpk2

III. 2. Genetic diversity obtained by BOXA1R primer

The primer BOXA1R produced clear, reproducible amplification pattern under the optimized PCR conditions, with a band range, 150 bp to 3.5 kb. A total of 14 polymorphic bands were scored (Fig. 4.12 A and B), and analyzed with UPGMA clustering using NTSYS. A dendrogram was constructed with a similarity index and genetic distance among the isolates. The dendrogram



A



B

Fig. 4.12 Fingerprints obtained by BOX-PCR for *M. phaseolina* , A. Lane no. 1 to 25 represents isolate no 1 to 25 and B. lane no 26 to 50 represents isolate no. 26 to 50 (Table 3.1), M-1 Kb DNA ladder.

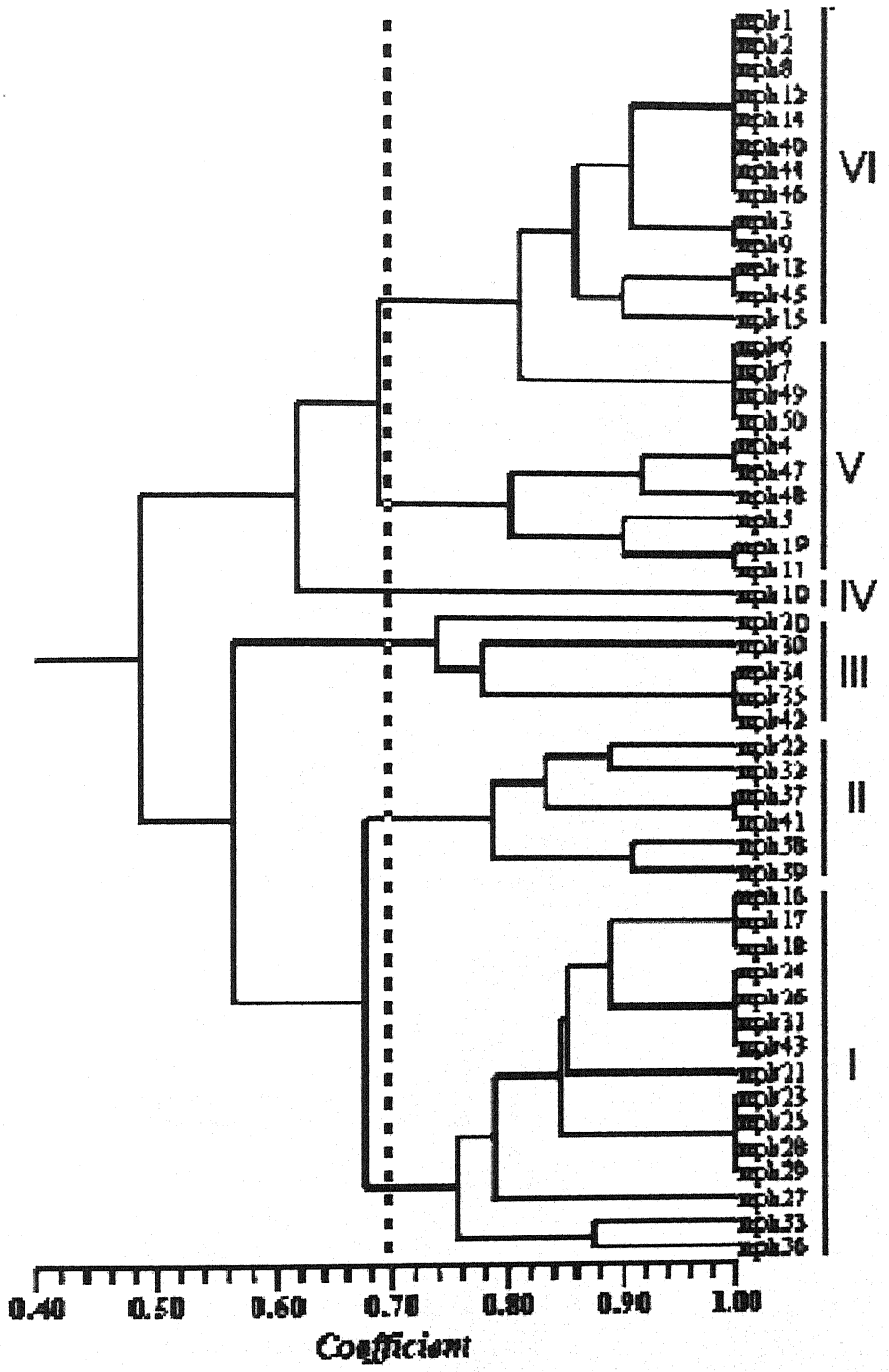


Fig. 4.13 UPGMA dendrogram showing the genetic relationship between fifty isolates based on the data obtained from the Box1R primer. The genotype clusters were indicated as I to VI. The details of isolates mpk1 to mpk 50 (Table 3.1). Doted line indicates similarity coefficient as a reference for clustering isolates.

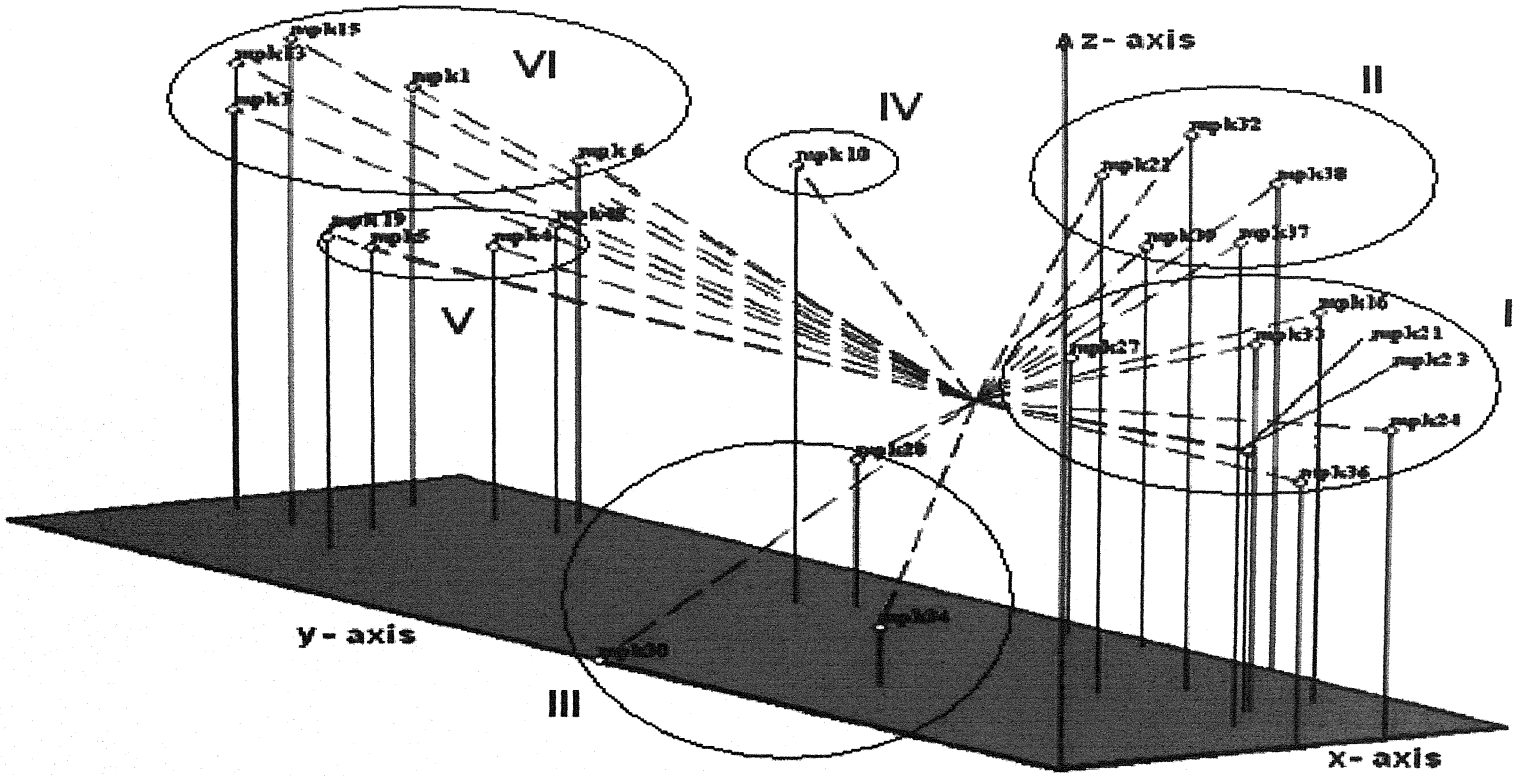


Fig. 4.14 Three-dimensional common principle coordinates analysis (CPCA) showing grouping of *M. phaseolina* isolates in to 6 genotypes (I to VI). One isolate has been selected from 100% similar genotypes as per UPGMA analysis of BOXA1R primer for CPC analysis, Doted lines represents eigenvectors of each component. Points on the top of solid lines represent the title of each isolate (Table 3.1).

sharing more than 70% genetic similarity coefficient were grouped all isolates into six major genotype clusters. In each cluster, isolates sharing 100% similarity coefficient were considered the same haplotype. Therefore, group I to VI were constituted by 7, 5, 3, 1, 5 and 4 haplotypes respectively (Fig. 4.13). Further; one isolate has been selected from 100% similar genotypes as per UPGMA analysis of BOXA1R primer for CPC analysis, the three-dimensional common principle coordinates analysis (CPCA) showing grouping of *M. phaseolina* isolates in to 6 genotypes (I to VI) (Fig. 4.14).

III. 3. Genetic diversity obtained by Oligo-9 primer

Twenty two isolates of *M. phaseolina* collected from Indogangetic region were subjected for genetic diversity by using 10-mer Oligo-09 random primer. The finger printing patterns were distinct from each other with a band range, 300 bp to 2.5 kb. A total of 14 polymorphic bands were scored (Fig. 4. 15), and analyzed with UPGMA clustering using NTSYS. A dendrogram was constructed with a similarity index and genetic distance among the isolates (Fig. 4.16).

III. 4. Genetic diversity obtained by URP 9F primer

Twenty-five isolates collected from three hosts such as sorghum (mpk4, 6, 16, 20, 21, 31, 39, 41, 42and 44), chickpea (mpk3, 5, 11, 23, 33, 38, 40 and 48) and soybean (mpk7, 9, 24, 27, 34, 35 and 36). DNA was amplified using the URPs (URP-9F and URP-P1F). The finger printing pattern by URP-9F produced band range, 320 bp to1.5 kb. A total of 15 polymorphic bands were scored (Fig. 4.17). URP-P1F was failed to produce distinct banding pattern. Dendrogram was constructed with the data obtained from URP-9F primer. UPGMA clustering analysis did not reveal any host-pathogen correlation (Fig. 4.18).

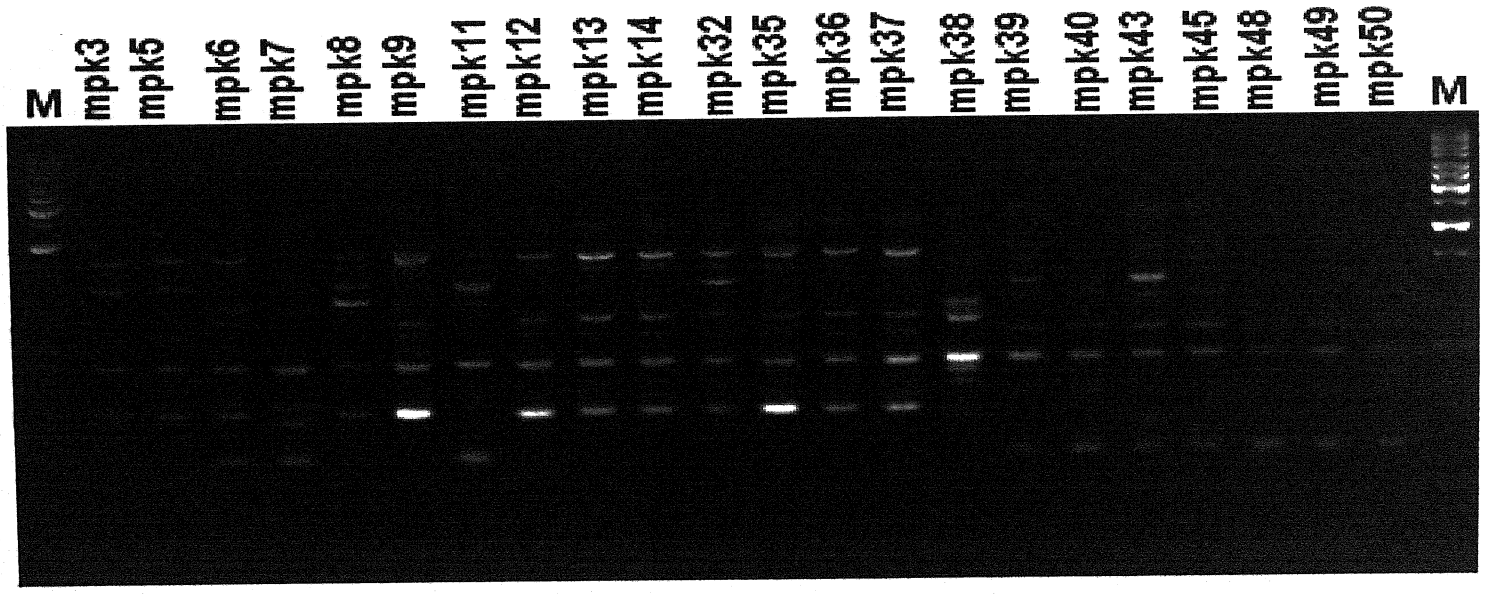


Fig. 4.15 RAPD-PCR fingerprinting: 22 isolates of *M. phaseolina* collected from Indo-Gangetic region of India were subjected to PCR amplification by using 10-mer RAPD primer Oligo-09. (A) Lane 1 to 22 indicating isolates of *M. phaseolina.* M-Represents1Kb ladder.

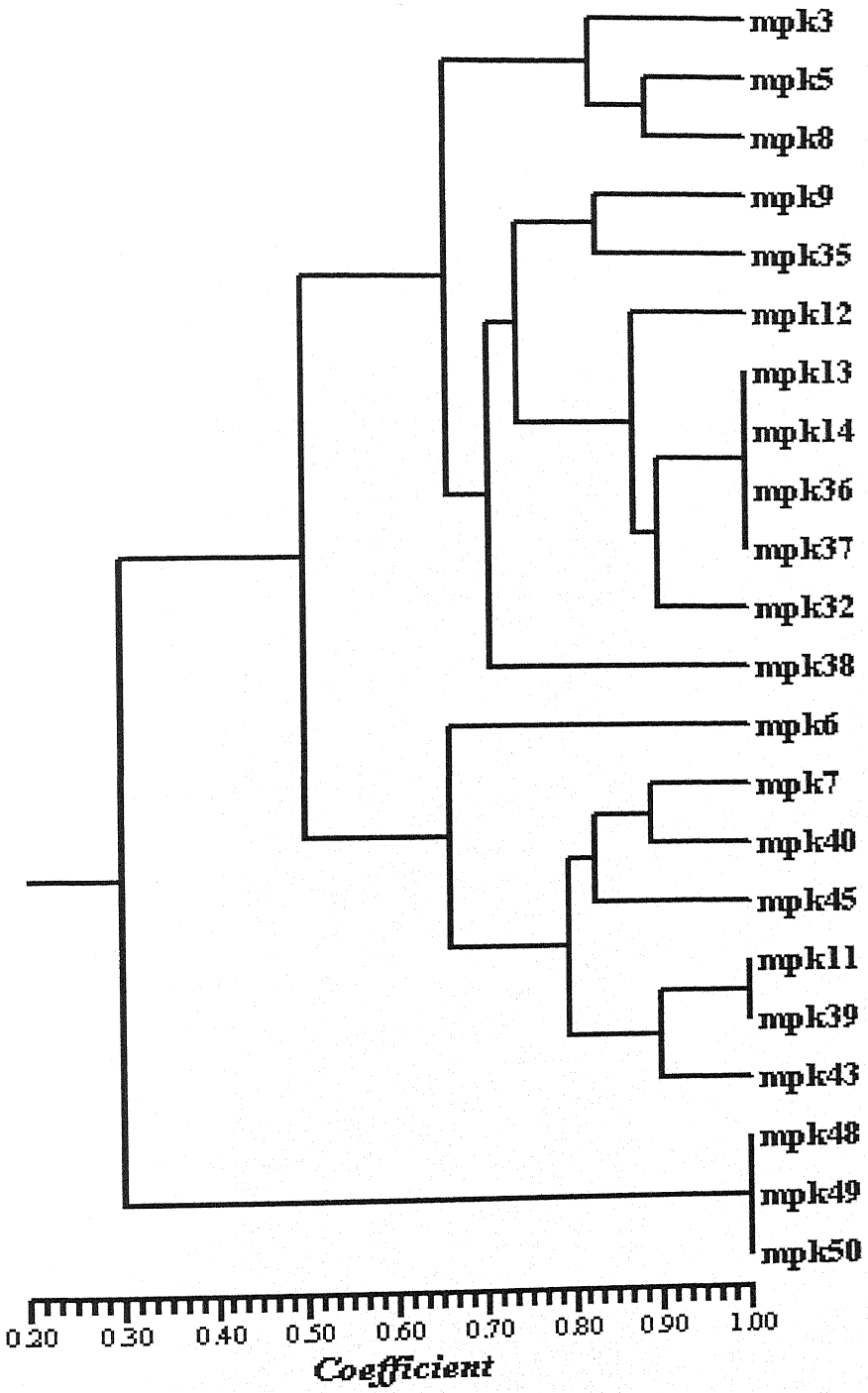


Fig. 4.16 Dendrogram showing: 22 isolates of *M. phaseolina* collected from Indo-Gangetic region of India As reveled by SAHN from UPGMA, based on RAPD data obtained by Oligo-9 primer.

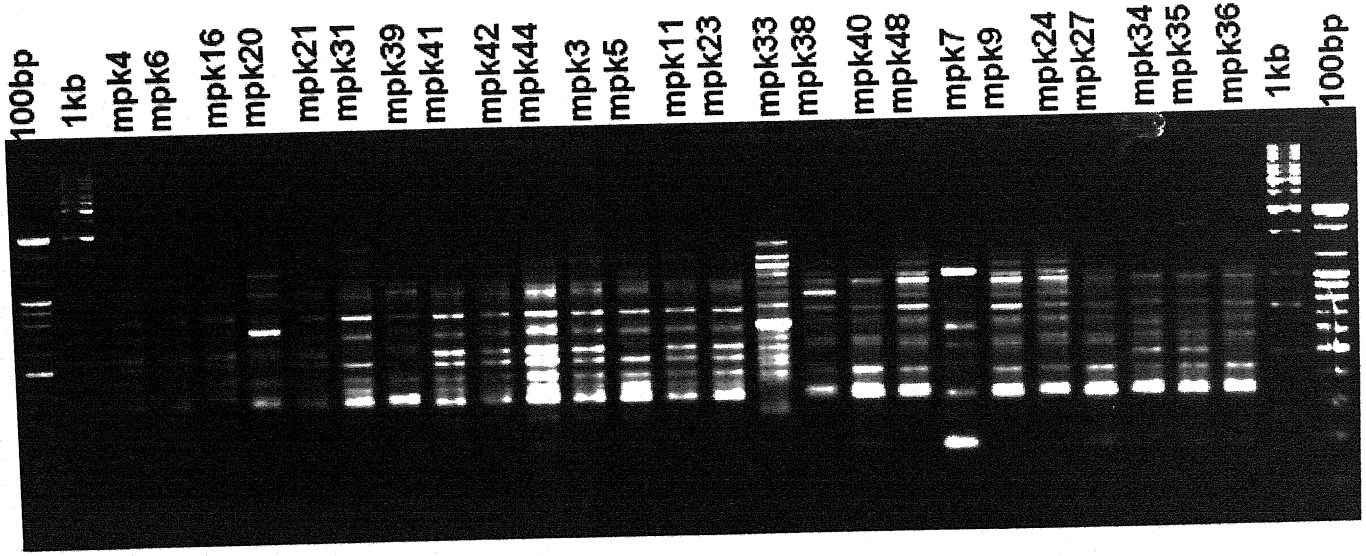


Fig. 4.17 *M. phaseolina* amplified by using URP 9F primer: Isolates mpk4, 6, 16, 20, 21, 31, 39, 41, 42 and 44 were from Sorghum, isolates mpk3, 5, 11, 23, 33, 38, 40, and 48 were from Chickpea and isolates mpk7, 9, 24, 27, 34, 35 and 36 represents Soybean. 100 bp marker and 1Kb ladder.

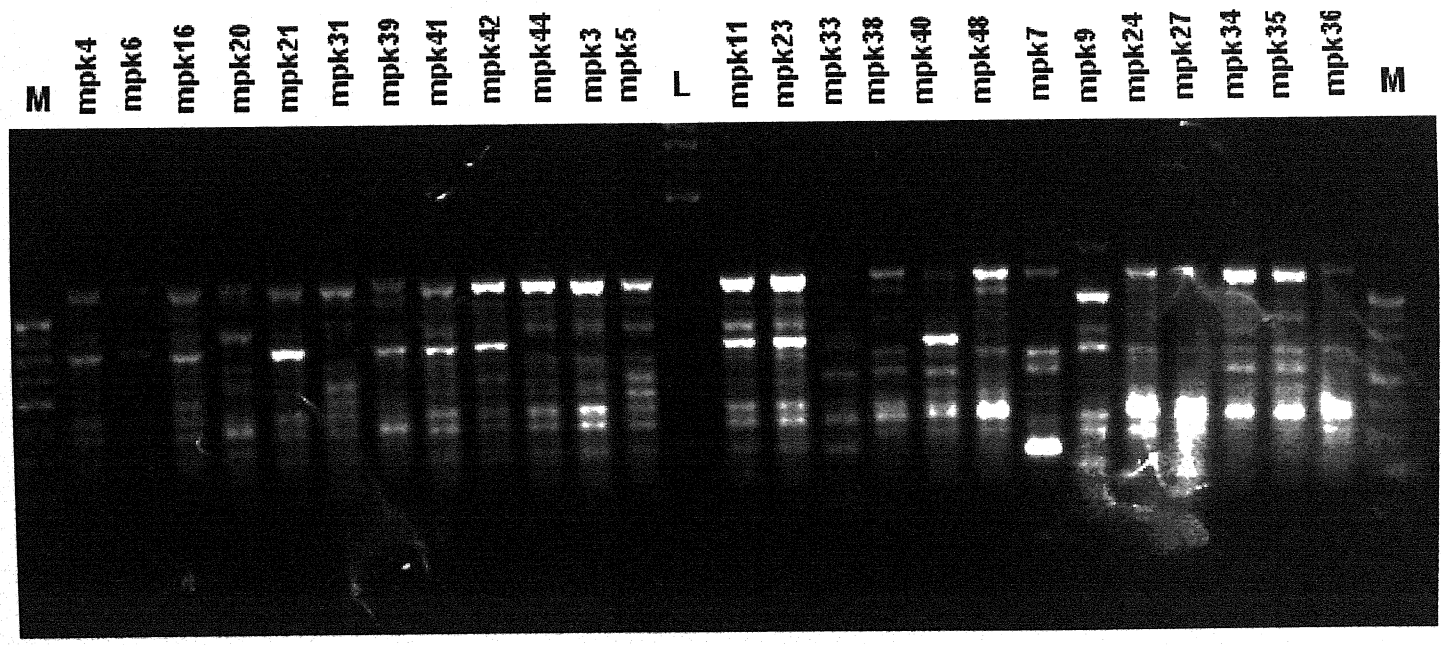


Fig. 4.19 *M. phaseolina* amplified by using M-13 mini satellite primer: Isolates mpk4, 6, 16, 20, 21, 31, 39, 41, 42 and 44 were from Sorghum, isolates mpk3, 5, 11, 23, 33, 38, 40, and 48 were from Chickpea and isolates mpk7, 9, 24, 27, 34, 35 and 36 represents Soybean. M- 100bp marker and L-1Kb ladder.

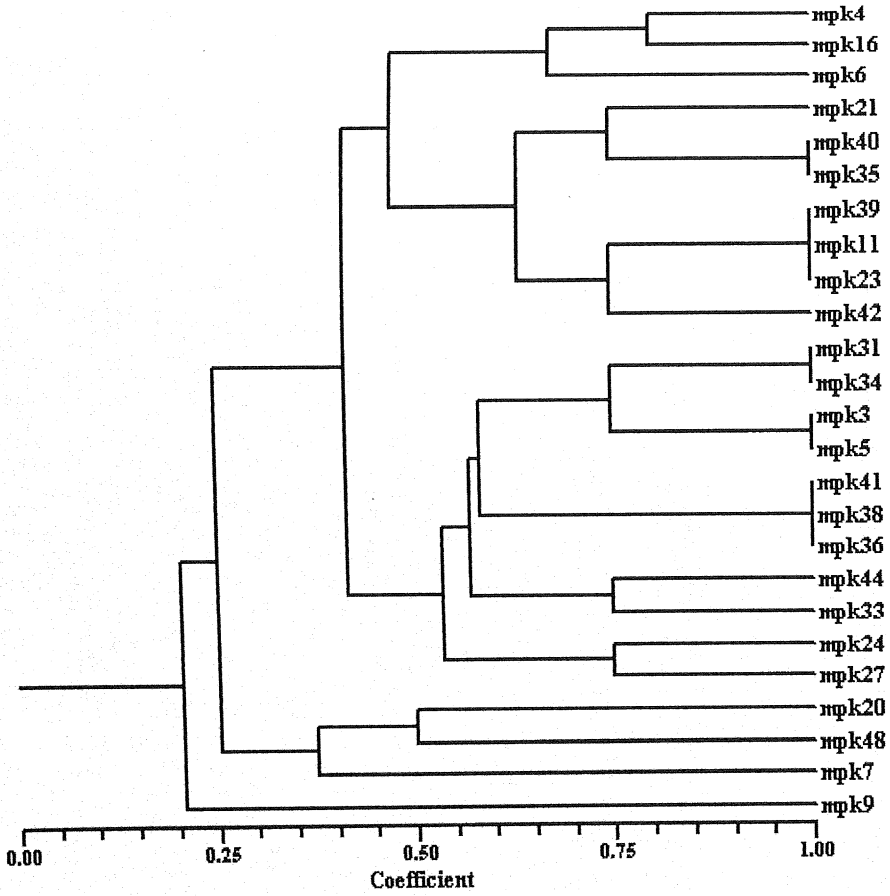


Fig. 4.20 Cluster analysis of *M. phaseolina* isolates collected from three different hosts (sorghum, chickpea and soybean). As reveled by SAHN from UPGMA, based on RAPD data obtained M13 mini satellite primer.

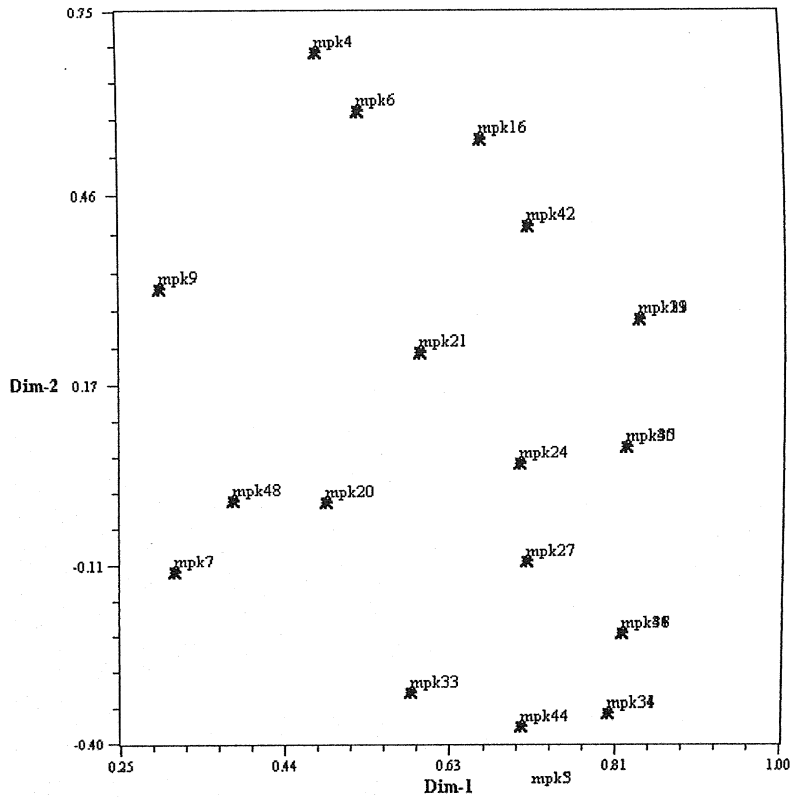


Fig. 4.21 Two-dimensional principal coordinates plot of 25 host-specific isolates of *M. phaseolina* based on differences in random amplified polymorphic DNA fingerprints obtained by using M13 mini satellite primer.

CHAPTER V

DISCUSSION

I. Morphological characterization and identification of *M. phaseolina*

II. Molecular characterization and identification of *M. phaseolina*

I. Morphological characterization and identification of *M. phaseolina*

The most challenging problem before the scientists and growers is to characterize, detect and identify different isolates of *M. phaseolina*. Until now, the characterization and identification of *M. phaseolina* is usually based on morphological criteria, though the morphological differences in *M. phaseolina* are minor with virtually no differences in sclerotial morphology but pathogenic isolates can be distinguished by differences in pathogenecity. Traditional detection from soil, seeds, diseased roots or the collar region is based on baiting techniques and culture on semi selective media (Cloud & Rupe, 1991). Root colonization can be monitored by estimating colony forming units per unit area of root, but this method is labor intensive, difficult to interpret, needs taxonomic expertise and requires at least 5-10 days.

All the isolates of *M. phaseolina* are morphologically very similar showing black/off-black colony structure (Fig. 4.1A and B). Even the sclerotium, which is assumed to enable this pathogen to overcome and survive the stress condition for extended periods, had no significant differences among different isolates. Earlier workers presented the information related to the "sclerotial-form" and "structure" by light microscopic studies (Reichert and Hellinger 1949; Townsend and Willetts 1954), and later on morphological characteristic of sclerotia was studied using electron microscope (Wyllie and Brown 1969).

Pathogenecity and pycnidium formation was different in all the isolates. Isolates originating from corn (family Poaceae) were least pathogenic as compared to that of other hosts (Table 4.1). The common pathogenecity of

isolates from soils implies that all propagules of *M. phaseolina* in the soil of an area must be considered potentially pathogenic to any crop planted. However, the variation among isolates in the pathogenecity experiment also implies that a soil population of *M. phaseolina* has a potentially wide range of pathogenecity phenotypes. What then are the possibilities of assessing the risk of yield reduction induced by charcoal rot when selecting a particular crop for planting in the field? Clearly the simple enumeration of pathogen propagules is unsatisfactory, as all propagules may not represent a single pathogenecity phenotype. Thus, there is an urgent need for a technique to rapidly evaluate the pathogenic potential of a large number of isolates.

Pycnidium formation was less in most of the pathogenic isolates. No relation was observed between the pathogenecity and pycnidium formation in various isolate. Test substratum was more significant determinate *M. phaseolina* for pycnidium production (Table 4.1).

Overall in this study, various morphological and cultural criteria were used to differentiate and characterize *M. phaseolina* isolates. However, it was difficult to differentiate isolates as: (i) all the isolates showed black-off-black colony structure, (ii) no significant difference in pathogenecity and pycnidium formation of *M. phaseolina* isolates were also observed. It was observed that morphological and cultural techniques were very tedious and time taking, particularly, if they lack reproductive structures. Even when structures of asexual reproductions are present, isolate may exhibit a typical, an intermediate, variable or no diagnostic morphological characteristic, which makes definite identification difficult.

II. Molecular characterization and identification of *M. phaseolina*

DNA sequencing and PCR fingerprinting are applied as common tools for phylogenetic studies (Kuhls *et al.* 1996; Kullnig-Gradinger *et al.* 2002; Meyer *et al.* 1992). DNA methods have brought additional valuable criteria to the taxonomy of plant pathogenic fungi (Hermosa *et al.* 2001; Lübeck *et al.* 2000) and phylogenetic classification (Kullnig-Gradinger *et al.* 2002; Lieckfeldt and Seifert 2000). Very few genetic studies have been made in the genus *Macrophomina.* The earlier preliminary work was mainly concerned with the characterization of different isolates, and efforts to recognize subgroups within the genus. Till to-date, no final taxonomic decision about the phylogenetic groupings have been made in the case of *M. phaseolina,* even though available molecular data strongly indicate that many sub-groups do represent in several isolates (Vandemark *et al.* 2000; Pecina *et al.* 2000).

The differentiation and characterization of *M. phaseolina* isolates belonging to different hosts plants on the basis of morphological and biochemical assay techniques was very tedious, time consuming and not very accurate. Therefore, this study was aimed towards developing rapid and accurate molecular tools to discriminate between isolates of different host plants. The various PCR based methods used in this study to generate fingerprint data from the various isolates using ITS region of rDNA, and application of well characterized random short sequences such as RAPD, and BOXA1R were evaluated.

Both the ITS and the IGS regions have been used to develop species-specific primers for plant pathogen detection *in vitro* and in plant material (*e.g.* Moukhamedov *et al.* 1993; Bridge and Arora 1998). It is becoming increasingly common in rRNA cluster studies to obtain sequences of all of the regions of

interest. Although knowledge of the complete sequences provides a large amount of information, useful information may be obtained from simple restriction digestions of rRNA amplification products. The rDNA sequence analyses, including internal transcribed sequences (ITS), have been used for taxonomic studies of many fungi (Kuhls *et al.* 1997; Gams and Meyer 1998; Hermosa *et al.* 2000; Lieckfeldt *et al.* 1999). In contrast to ITS, fewer studies have been made using IGS region for genetic characterization. Arora *et al.* (1996) used RFLPs derived from the IGS, located between the rRNA gene clusters, to determine variability within the species *V. chlamydosporium*, and also other closely related species. Studies on the IGS region for the characterization of different fungi were also undertaken by Appel and Gordon (1995), Kim *et al.* (1992), Morton *et al.* (1995). There are also reports that non-transcribed sequences like IGS, allowed discrimination at intra-specific level between different strains of *Laccaria* species (Henrion *et al.* 1992) and *Fusarium oxysporum* (Edel *et al.* 1995).

In the present study ITS region has been targeted to study variability among the isolates of a single species and also to develop species-specific probes and primers (Edel *et al.* 2000; Gardes and Bruns 1993; S. Oh *et al.* 2003). ITS-RFLP analysis of *M. phaseolina* isolates collected from diverse geographical locations revealed little variability within the species. The restriction endonucleases employed either produced identical patterns or had multiple cleavage sites giving small fragments that were difficult to analyze on agarose gel and also lack reproducibility (Lovic *et al.* 1995; Schmidt and Moreth 1999). In addition, restriction enzymes like *Apa* I, *Hin*d III and *Sac* I did not exhibited any cleavage site in the amplified ITS fragment of *M. phaseolina*. Similar results have been reported by other workers (Arora *et al.* 1996; Su *et al.* 2001). In a recent study

some degree of polymorphism in restriction patterns of the ITS region, including part of 25S rDNA have been reported in *M. phaseolina* (Purkayastha *et al.* 2006). Since, ITS-RFLP could not detect variations within different isolates in our study; we resorted to sequencing of eight isolates collected from different hosts and diverse ecological conditions. Alignment of these sequences along with two sequences from the GenBank revealed various conserved and variable regions in the ITS sequences of *M. phaseolina* (Fig. 4.5). For the better understanding of these regions, the amplified ITS sequence was virtually divided into five regions. Region 4 (not shown in the Fig. 4.5) was deleted as it contained sequences that showed variations among the isolates of *M. phaseolina*. Further, sequence alignment with other closely related genera of soil borne fungi helped us to identify two regions that were conserved among the isolates of *M. phaseolina* but exhibited a high degree of variability among isolates of other genera. Similar approach has been used earlier to specifically detect *F. oxysporum* on the basis of sequence variation in 28S rRNA gene (Edel *et al.* 2000). The two primers designed from these selected regions of ITS showed specificity in the PCR assays. Optimization of the PCR conditions and validation of primers yielded a specific amplicon of 350 bp for *M. phaseolina* isolates (Fig. 4.6). The absence of 350 bp product in other species of soil borne fungi, bacteria and actinomycetes confirmed that the primers can be selectively utilized to identify *M. phaseolina*. The probe was also shown to be specific for *M. phaseolina* and no signals were obtained with non-specific target ITS sequences. This is the first report on development of specific primers and probe for the identification and detection of *M. phaseolina in vitro* conditions.

The genetic characterization of prevailing plant pathogenic *M. phaseolina* is of importance for the effective disease management. One of the oldest and most

widely used PCR method is Random amplified polymorphic DNA (RAPD) analysis (Welsh and McClelland 1990; Williams *et al.* 1990). Essentially RAPD analysis relies on the reduction in specificity of the PCR process at reduced temperatures. Total genome DNA extractions are used, and these are amplified with single, short (usually decamer) primers at a reduced annealing temperature. These conditions result in less stringent binding of the primer to the target DNA, and allow the amplification of a number of generally small regions of DNA. In many cases these studies have shown correlations between band patterns and host, disease type or geographical origin. Band patterns were also used to differentiate between forms of the same fungus causing different disease symptoms (Pei *et al.* 1997).

Single-strand conformation polymorphism (SSCP) analysis is a powerful tool, which can detect single base mutations or variations (Orita *et al.* 1989; Rubio *et al.* 1996; Kong *et al.* 2000; Sambrook and Russell 2001). A protocol for SSCP analysis of ribosomal DNA for species separation within the genus *Phytophthora* was reported recently (Kong *et al.* 2003). This study provides additional evidence that SSCP analysis is a powerful tool for detection of nucleotide variations. The different SSCP patterns were obtained for isolates of *M. phaseolina* that are morphologically similar. Three different types of banding pattern indicate the 5.8S rDNA may be because of single nucleotide polymorphism at more than one position.

In the present study, examination of a large number of *M. phaseolina* isolates were studied for genetic diversity, host specificity and geographical origin by using molecular markers indicating significant genetic diversity among the isolates collected from 8 major agro climatic zones in India. Various studies

devoted to the genetic, geo-diversity and pathogenic variability of *M. phaseolina* collected from Mexico and other countries, has found clear differentiation (Vendemark *et al.* 2000; Mayek-perez *et al.* 2001; Pecina-Quintero *et al.* 2001; Su *et al.* 2001). Jana *et al* (2003) developed taxonomic marker for population studies by using a single RAPD primer that distinguishes isolates of *M. phaseolina* from soybean, sesame, ground nut, chickpea, cotton, common bean and 13 other hosts. Among the Indian population there are no reports available on correlation between the genotype and geographical or biological origin. Recently, sorghum isolates of Indian origin were distinguished based on chlorate sensitivity (Das *et al.* 2007). Significant variations among the pathotypes obtained from different continents have also been reported (Reyes-Franco *et al.* 2006).

RAPD analysis has been useful tool for detecting inter-specific genetic variation in *M. phaseolina* population in different countries (Fuhlbohm 1997; Su *et al.* 2001; Alvaro *et al.* 2003). Using the tool it was observed to have considerable variation among the *M. phaseolina* isolates obtained from different geographical locations and hosts. Although very less genetic variation among the Indian isolates from sorghum have been reported by Das *et al.* (2007). Purkayastha *et al* (2006) has found some degree of genetic diversity and pathogenic variability among the Indian isolates with the help of ITS-RFLP and RAPD. However, the presence of two monomorphic bands among the isolates irrespective of geographical or biological origin indicates genetic similarity among the isolates. In absence of sexual reproduction in *M. phaseolina* genetic variability could occur by slow recombinant process like fusion of cells or parasexual recombination (Carlile, 1986). The presence of two monomorphic bands suggests that isolates might have evolved from a common ancestor but due to geographical isolation followed by natural selection and genetic drift might have segregated in to

subpopulations. Thus the clustering of genotypes in to geographical clusters is an indication of adaptation of isolates over the years to particular location.

Thus RAPD-PCR could be a useful tool for rapid typing of isolates of *M. phaseolina*. By using these motif primers, plant pathologists may be able to identify heterogeneity within the subgroups of isolates that originated from the same locations/host plants, indicating that specificity of identification can be achieved by using RAPD fingerprinting. Another common PCR fingerprinting method that has been used for plant pathogenic fungi is amplification of sequences based on simple repetitive primers (Versalovic *et al.* 1998). In this method, single short repetitive primers are used at moderate annealing temperatures in order to amplify largely repetitive fragments of the genome. In this study, repetitive elements that are highly conserved in fungal as well as in bacterial kingdom such, BOXA1R sequences were also present in *M. phaseolina*. The distribution of these elements was variable among *M. phaseolina* isolates, which allowed the differentiation of the isolates. Both BOXA1R and M13 mini-satellite PCR techniques produced reproducible results, especially with purified genomic DNA as a template, when the primer concentration and composition of buffer were strictly controlled. The dendrogram constructed by BOXA1R-PCR fingerprinting (Fig. 4.12A and B), that clearly divided all the *M. phaseolina* in to six major groups with 70% similarity.

Therefore, this technique is useful for rapid and routine genetic diversity analysis from large number of isolates representing similar phenotypic characteristics. Further, the UPGMA dendrogram and two-dimensional CPCA analysis of M13 mini-satellite primer and URP-9F did not revealed any

differentiation among the host-specific isolates from soybean, chickpea and sorghum (Fig. 4.20). At this point, it is important to mention that host specialization with *M. phaseolina* was first described by Pearson *et al* (1986) suggesting that isolates from one specific host are more suited to colonize. Later, Could & Rupe (1991) working with isolates of soybean and sorghum, also observed differences in pathogenecity. Su *et al* (2001) grouped isolates of soybean, sorghum and cotton based on their host origin.

The objective of this research was to propose a simple procedure for the characterization and identification of isolates belonging to different hosts and diverse agro climatic regions. DNA polymorphic patterns, in general, indicate that *M. phaseolina* isolates are closely related but they can be differentiated genetically. The RAPD, BOXA1R, and M13 polymorphism supports the concept that *M. phaseolina* isolates are genetically different. The isolates of *M. phaseolina* clearly showed that the isolates belonging to chickpea exhibited maximum dissimilarity with other isolates. The fingerprinting patterns, obtained by using these motif gene sequences are simple alternatives to ITS-RFLP determination, where a large number of endonucleases are used to obtain polymorphic patterns. We have compared the general utility of ITS, RAPD, BOXA1R and M13 primers in differentiating the isolates of *M. phaseolina* which enabled grouping of closely related strains with simple banding patterns. However, in contrast to ITS, the random motif primers generated complex patterns which were often difficult to analyze and thereby to quantify variations between isolates. As observed by other workers (Edel *et al.* 1995), this study also revealed prominent and faint bands in BOXA1R and M13-PCR, which were not always amplified to the same extent. However, prominent bands were found to be reproducible and consistently present, and provided a sufficient level of discrimination within the

closely related strains. Thus, using the five different primer sets instead of just one, gave a broader picture of the diversity and similarity among *M. phaseolina* isolates. The technique is ideal for a large number of species, populations or pathotypes that need to be discriminated without knowledge of DNA sequences. For plant pathological and epidemiological studies, the accurate taxonomical grouping is a key factor for the identification of *M. phaseolina* isolates, which will lead to the understanding of their genetic biodiversity in the natural environment.

In conclusion, characterization, detection and identification of *M. phaseolina* based on the visual examination of morphology is highly selective, and isolate-specific identification of this fungus is difficult. Molecular techniques present several advantages over the traditional ones. As these techniques do not rely on phenotypic examination, gene expression is not required and identification time can be reduced significantly. As noted in this study, the molecular markers can be derived from both variable and conserved regions of the nuclear and rDNA, and some of these could be used to define populations at all levels from an individual isolate. Further, in this study we developed species-specific olegonucleotide primers and probe for identification and detection of *M. phaseolina*. The speed, accuracy, and reliability of detection methods were greatly enhanced by optimizing the DNA extraction protocols; or by using internal standard DNA in the PCR reaction. These markers could be useful to study the epidemiology of these important plant pathogenic fungi in field conditions, which in turn, may lead to develop better disease control strategies. The hybridization techniques were used for direct detection of fungi, although novel developments in this field, including the application of DNA probes and microarray techniques are encouraging.

Detection of fungal pathogens, such as *M. phaseolina*, which cause diseases to 400 economically important plants, is still a time-consuming method particularly in the case of large-scale testing. The increasing availability of RT-PCR based molecular kits for the detection is a step toward standardization of the molecular techniques. The development of diagnostic kits would be a step ahead for the authorization as one of the accepted technique for quarantine and plant protection agencies. The work presented in this piece of research is just a beginning. Much collaborative efforts are needed before a diagnostic kit for this important plant pathogen could be made available commercially.

SUMMARY

SUMMARY

Macrophomina phaseolina (Tassi) Goid. is one of the most important plant pathogenic fungi. It is primarily a soilborne pathogen with wide distribution range, varied host plants, greater longevity higher competitive saprophytic ability, and persists even under diverse environmental conditions. As a root/soil inhabitant, the fungus is widespread in warmer areas, invades immature, damaged or senescent tissues of the root/stem cortex. Plants are generally attacked at seedling/flowering stages when conditions are hot and dry. *M. phaseolina* causes many important diseases like damping-off, seedling blight, collar rot, stem rot, charcoal rot and root rot in various economically important crop plants. The fungus belongs to subdivision Duteromycotina, class Coelomycetes. This genus has only one species. The biggest challenge before the plant pathologists is to detect/identify different isolates of *M. phaseolina*, which appears morphologically very similar. Until now, the characterization and identification of isolates of *M. phaseolina* is usually based on morphological criteria. In general, there is a great need for the development of direct identification and detection methods for this soilborne pathogenic fungus. The main aim is to develop a rapid, sensitive, robust, and specific method that can be used directly on diseased plants is of profound importance for the plant pathologists. This will enable intervention with appropriate measures to prevent serious economic losses. The hybridization techniques have only been used for direct detection of fungi, although novel developments in this field, including the application of DNA probes and microarray techniques are encouraging. Amplification-based methods are frequently applied for detection of pathogens. Most PCR-based detection methods target part of the ribosomal RNA gene cluster, although RAPD markers are also widely used. For the detection of toxin

producing fungi, the most direct procedure is targeting of the toxin biosynthetic genes. Such approaches have been used recently for the detection of a number of fungi-producing toxins such as ATs, patulin, PR toxin, and trichothecenes. The recent development of real-time PCR methodology has made it possible to quantify the amount of organisms in infected plants.

Morphological Characterization and Identification of *M. phaseolina*

All isolates of *M. phaseolina* form black/blackish-ash colour colony. The sclerotial shape in most cases was irregular, while in few cases they are long to elongate. Mycelium is usually septate hyaline at first, and later changed to black in colour. The isolates colonizing/causing root rot/collar rot on beans, chickpea, corn and soybean were selected for the present study. The sclerotia obtained from the above host plants exhibited no apparent morphological differences. In this study, efforts were made to characterize the *M. phaseolina* isolates on the basis of pathogenecity, pycnidium formation and utilization of chlorate. The characterization of different isolates, on the basis of pathogenecity and pycnidium formation, was very difficult and confusing. Though our results showed that corn isolates were least pathogenic as compared to pathogenic isolates of other hosts, the pycnidium formation did not show any relation with the pathogenecity.

Overall in this study, various morphological and cultural criteria were used to differentiate and characterize *M. phaseolina* isolates. However, it was difficult to differentiate the isolates because: (i) all the isolates showed black/off-black colony structure, (ii) the sclerotia also did not exhibit any significant difference between the isolates, and (iii) no significant difference in pathogenecity and pycnidium formation. It was noticed that morphological and cultural techniques were very tedious and time taking, particularly, if they lack reproductive structures as in the case of *M. phaseolina*. Even when structures of asexual

reproductions are present, isolate may exhibit a typical, an intermediate, variable or no diagnostic morphological characteristic, which makes definite identification difficult. Therefore, identification and characterization of *M. phaseolina* isolates based on morphological and cultural criteria is very tedious, confusing, time consuming and not very accurate.

PCR- based identification, detection and characterization of *M. phaseolina*

Very few genetic studies have been made in the genus *Macrophomina*. DNA methods have brought additional valuable criteria to the taxonomy of plant pathogenic fungi; phylogenetic classification, DNA sequencing and PCR fingerprinting are applied as common tools for phylogenetic studies. To-date, no final taxonomic decisions about the phylogenetic grouping have been made in the case of *M. phaseolina*, even though some available molecular data strongly indicate that many sub-groups do represent in several isolates. To-date, genes involved in pathogenecity of *M. phaseolina* have not been sequenced and characterized, nor no gene expression analysis has been reported. In this study, PCR-based molecular methods were used for the detection characterization of different isolates. The rDNA region such as internal transcribed spacers (ITS) was targeted for identification and detection of various isolates of *M. phaseolina*. The ITS consists of two non-coding variable regions that are located within the rDNA repeats between the highly conserved small subunit, the 5.8S subunit and the large subunit rRNA genes. Other motif primers such as random amplified polymorphic DNA (RAPD), BOX repetitive sequences and M13 mini-satellite sequences were also used. The RAPD fingerprinting assay detects small inverted nucleotide sequence repeats throughout the genomic DNA.

DNA amplification of *M. phaseolina* isolates by using universal primers ITS1 and ITS4, which yielded a fragment of approximately 650 bp. The amplified

region was digested with ten different restriction enzymes. The restriction enzymes *Eco*R I, *Sau* 3A I, *Alu* I and *Cla* I produced identical pattern for all the isolates. The enzymes *Hap* II, *Msp* I and *Taq* I have multiple cleavage sites in ITS amplified product and produced fragments less than 100 bp in size. However, the enzymes *Apa* I, *Hin*d III and *Sac* I failed.

Sequence comparison of ITS region encompassing ITS-1, 5.8S rRNA gene and ITS-2 of *M. phaseolina* and other related fungal species revealed three regions that were conserved among the *M. phaseolina* isolates. Two primers MpKFI and MpKRI were designed from the specific nucleotide areas and one oligonucleotide probe MpKH1 (19-mer) was designed from the conserved region, adjacent to 5.8S gene showed in the region 3. The designed primers yielded single amplified product of 350 bp with all the *M. phaseolina* isolates. The specificity of the primers was tested on representative species of common soilborne fungi, bacteria and actinomycetes. The primer pair was found to be specific for *M. phaseolina* as none of the other microbes yielded amplified products under the identical conditions. Probe MpKH1 selectively hybridized with strains of *M. phaseolina,* but failed to do so with the representative strains of different groups of soilborne microbes. The 5.8S rDNA region of *M. phaseolina* was amplified by specific primers; the resultant amplicon was subjected for SSCP analysis. It was found be single nucleotide polymorphism at 5.8S region within *M. phaseolina* isolates.

In the present study RAPD markers were used for the examination of a large sample of *M. phaseolina* isolates for genetic diversity, host specificity and geographical origin. Thirteen RAPD 10-mer primers, (OPA-15, OPA-11, OPA-10, OPB-08, OPV-17, OPC-10, OPB-07, OPB-06, OPD-11, OPB-17, OPE-01, Oligo-09 and OPA-09), two URPs (URP-9F and URP-P1F), one rep-primer (BOXA1R) and one mini-satellite primer (M13) were analyzed. Dendrogram were constructed

for the amplicons to different RAPD primers. Primer OPB-08 exhibited 18 amplified bands (100 bp to 3 Kb), indicated the significant genetic diversity among the isolates collected from 8 major agro climatic zones in India. Results from RAPD analysis showed that *M. phaseolina* isolates were clearly differentiated from each other based on their geographical origin, which suggest that the adaptation towards the geographical conditions has been developed up to certain extent. The dendrogram pattern, which clearly delineates sub-populations that can be explained in terms of adaptation of isolates to their hosts in that populations recovered from specific location, may be subjected to similar selection pressures. Similarly, the dendrogram obtained by single short repitative primer (BOXA1R), clearly divided all the *M. phaseolina* into six major groups with 70% similarity. Therefore, this technique can also be used for rapid and routine genetic diversity analysis from large number of isolates representing similar phenotypic characteristics. Further, the UPGMA dendrogram and two-dimensional CPCA analysis of M13 mini-satellite and UPGMA analysis of URP-9F primers did not revealed differentiation amongst the host-specific isolates from soybean, chickpea and sorghum.

In conclusion, the RAPD polymorphism supports the concept that *M. phaseolina* isolates are genetically separated. The fingerprinting patterns, obtained by using these motif gene sequences are simple alternatives to ITS-RFLP determination where a large number of endonucleases are used to obtain polymorphic patterns. This can be an ideal technique for a large number of species, populations or pathotypes of *M. phaseolina* belonging to different host plants that can be discriminated without any knowledge of DNA sequences.

Thus, it is clear that characterization, detection and identification of *M. phaseolina* isolates on the basis of visual examination of morphology is highly

selective, and isolate-specific identification of this fungus is difficult. The biochemical and immunological techniques possesses many advantages, but have some serious limitations due to their specificity. These techniques are also tedious and time consuming. Molecular techniques present several advantages over the traditional ones. As these techniques do not rely on phenotypic examination, gene expression is not required and identification time can be reduced significantly. As noted in this study, the molecular markers can be derived from both variable and conserved regions of the nuclear and rDNA, and some of these could be used to define populations at all levels from an individual isolate upwards.

Further, studies are needed to develop specific molecular markers for different *M. phaseolina* isolates, and primers developed from a variety of DNA sequences including specific regions such as genes coding for rRNA *etc.* These markers could be useful for the study of epidemiology of this important plant pathogenic fungus in field conditions, which in turn, may lead to the development of our understanding for better disease control strategies.

Sensitivity of a molecular method depends not only on the detection system, but also to a great extent on the nature of fungi. In order to increase the sensitivity, adequate protocols for the different *M. phaseolina* isolates *in situ* conditions have been established *i.e.*, speed, accuracy, and reliability of detection methods were greatly enhanced by optimizing the DNA extraction protocols, PCR and hybridization conditions.

Detection of fungal pathogens, such as *M. phaseolina*, which cause diseases to 400 economically important plants, is still a time-consuming method particularly in the case of large-scale testing. The increasing availability of RT-PCR based

molecular kits for the detection is a step toward standardization of the molecular techniques. The development of diagnostic kits would be a step ahead for the authorization as one of the accepted technique for quarantine and plant protection agencies.

The work presented in this piece of research is just a beginning. Much collaborative efforts are needed before a diagnostic kit for this important plant pathogen could be made available commercially.

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APPENDIX

APPENDIX-I

DNA isolation by Lysis Buffer Protocol

Harvest 3-4 days old mycelia

↓

Add 500 µL of Lysis extraction buffer (pH-7.2)

↓

Crush with frozen mortar-pestle

↓

Incubate tubes at 65°C for 1 hour with occasional mixing by gently inverting the tubes

↓

Add equal volume of water-saturated phenol

↓

Shake for 5-10 min

↓

Centrifuge at 13,000-15,000 rpm for 15 min at 4°C

↓

Extract with Water saturated phenol: $CHCl_3$: Isopropyl alcohol (25:24:1)

↓

Mix by inverting the tubes 10 times very gently

↓

Centrifuge samples at 15,000 rpm for 15 min at 4°C

↓

Transfer the upper phase with the help of cut tips to a new tube

↓

To this upper phase, add, 1/4th part $CHCl_3$ + 1/10th part Sodium acetate

↓

Add Isopropanol 0.7 times of the total volume of the upper phase

↓

Mix by inverting the tubes 10 times very gently

↓

Centrifuge at 15,000 rpm for 15 min at 04°C

↓

Remove aqueous phase, wash with 70% ice-cold ethanol (for more than half an hour)

↓

Decant the liquid material

↓

Dry the pellet at room temperature or in a 37°C incubator & add 200 µL of TE buffer

↓

Store DNA in small aliquots at 4°C

Bioinformatics tools used in this study

Editing of the raw sequence	Chromas
Sequence comparison/analysis	FASTA, BLAST
Sequence submission at NCBI Genbank	Sequin, BankIt
Sequence retrieval from Genbank and storage	BLAST, FASTA
Multiple sequence alignment and editing	Clustal X, Genedoc, BioEdit
Designing of primers and probe	Primer3, GeneRunner
In silico analysis and testing of specificity of Primers and Probe	GeneRunner, BLASTn

APPENDIX-II

Potato Dextrose Agar (PDA)

Potato	200 g
Dextrose	20 g
Agar agar	17 g
pH	5.6
*Streptomycin sulphate	25µg / ml
dH_2O	to 1000 mL

Lysis Buffer

Tris HCl	50 mM
EDTA	50 mM
SDS	3%
2-mercapto ethanol	1%
pH	7.3

TE Buffer

Tris HCl	10 mM
EDTA	1 mM
pH	8.0

TBE Buffer (5X)

Tris base	54
EDTA	20 ml (0.5 M)
Boric acid	27.5
pH	8.0

Gel loading Buffer

Bromophenol blue	0.25
Xylene cyanol	0.25
Glycerol	30 mL
Water	70 mL

40% Acrylamide/Bis (37.5:1)

Acrylamide	38.93 g
Bis-acrylamide	1.07 g
dH_2O	to 100 mL

Filter through a 0.45 µ filter and store at 4 °C.

10% Ammonium Persulfate

Ammonium persulfate	0.1 g
dH_2O	1.0 mL

Store at -20 °C for about a week.

2x Gel Loading Dye

Bromophenol blue	0.05 g
Xylene cyanol	0.05 g
Formamide	9.5 mL
0.5 M EDTA, pH 8.0	0.4 mL
Total volume	10.0 mL

Store at room temperature.

10x TBE Buffer

Tris base	108 g
Boric acid	55 g
0.5 M EDTA, pH 8.0	40 mL
dH_2O	to 1 L

Mix. Autoclave for 20–30 minutes. Store at room temperature.

Reagent	6% Gel	8% Gel	10% Gel	12% Gel
40% Acrylamide/Bis	6 mL	8 mL	10 mL	12 mL
10x TBE	4 mL	4 mL	4 mL	4 mL
TEMED	40 µL	40 µL	40 µL	40 µL
10% Ammonium persulfate	400 µL	400 µL	400 µL	400 µL
dH_2O to	40 mL	40 mL	40 mL	40 mL

Add dH_2O to 40 mL and mix. Cast the gel immediately after adding the TEMED and ammonium persulfate.

LIST OF PUBLICATIONS

Babu. KB., Srivastava, A. K., Saxena, A. K and Dilip K. Arora (2007) Identification and detection of *Macrophomina phaseolina* by using species-specific oligonucleotide primers and probe. **Mycologia. 99(6), 733-739.**

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